Analysis of HIV-1 specific antibodies induced by Ugandan viruses or virus encoded peptides

Thesis

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Analysis of HIV-1 specific antibodies induced by Ugandan viruses or virus encoded peptides

by

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ABSTRACT

The main objectives of the work presented in this thesis were to develop an assay to subtype viruses circulating in Uganda and to study virological and immunological factors that might be important in HIV-1 vaccine development in East Africa.

Development of an antibody binding assay for serological subtyping revealed a complex pattern of cross-reactivity, and led to further investigation of these responses. Competitive inhibition and adsorption studies identified a spectrum of antibodies in the human antisera, possibly due to multiple immune responses to more than one viral epitope or to one immunodominant epitope. Neutralizing antibodies to specific V3 loop peptides were raised in rabbits; MN and U31 peptides were good immunogens, but Ugandan consensus sequence peptides raised antisera with weak peptide reactivities. Antibody binding assay of partial V3 loop peptides indicated that most Ugandan antisera recognised an epitope(s) in the C-terminal region of the V3 loop, not at the apex.

Virus samples from the UVRI clinic and the Natural History cohort in rural SW Uganda were isolated and characterized. Comparison of primary isolates in PBMC cultures and cell adapted variants indicated that SI and NSI variants may be present within a primary isolation culture.

Neutralizing antibody titres were compared between cell line adapted viruses and primary isolates: Differences found led to the use of low passaged primary isolates for this investigation. Cross-clade neutralizing antibodies were present in most antisera, many with high neutralization titres (>640); suggesting a shared immunogenic epitope, which could be important in vaccine design. Analysis of

sequential serum samples revealed no decrease in neutralizing titres with time. One sequential virus isolate (3029), re-isolated after 2 years, was more resistant to neutralization by heterologous antisera. No correlation was established between antibody binding and neutralization by human or rabbit antisera.

Comparison of clinical data with neutralization data was inconclusive.

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I dedicate this work to Harambee Kianga.

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1. INTRODUCTION

1.1 The Epidemic of AIDS

Human Immunodeficiency Virus Types 1 and 2 (HIV-1 and HIV-2) are the causative agents of Acquired Immune Deficiency Syndrome (AIDS). This syndrome was first recognised in 1981 in the United States of America when homosexual men presented with a variety of diseases, especially Kaposi's sarcoma and *Pneumocystis carinii* pneumonia which were attributed to a form of immunodeficiency (Center for Disease Control, 1981). At this time the life style of these predominantly young men was thought to be responsible for their immunodeficiency. However, by 1982 reports of AIDS were registered in other groups, including intravenous drug users and haemophiliacs (Center for Disease Control, 1982). In 1983 a similar syndrome was reported in African people living in Europe (Brunet *et al.*, 1983) soon followed by the first reports of African AIDS in Rwanda and Zaire (Piot *et al.*, 1984). This emerging pattern of disease launched the search for an infectious agent. Clinical examination of infected Americans revealed a reduced T helper lymphocyte level with increased suppressor T cells. In 1981 human T cell lymphotropic virus (HTLV-1) had been discovered by Gallo: this retrovirus, which causes adult T cell leukaemia, was the first virus shown to infect human T cells. Methods of T lymphocyte culture devised by Gallo and his colleagues were invaluable in the race that ensued to identify the causative agent of AIDS. In France, Barré-Sinoussi, Chermann and Montagnier discovered lymphadenopathy-associated virus (LAV) (Barre-Sinoussi *et al.*, 1983) and in America, Gallo's team isolated a virus they designated human T cell lymphotropic virus III (HTLV-III) (Gallo *et al.*, 1984). Both of these new retroviruses were isolated from AIDS patients' lymphoid tissue and were propagated on CD4+ immortalised T cell lines (Heymann *et al.*, 1993). In 1986 it was agreed to rename these retroviruses HIV-1 (Coffin, 1986; Coffin *et al.*, 1986).

A second human immunodeficiency virus (HIV-2) was isolated by Clavel and colleagues in 1986 from two West African AIDS patients who had repeatedly

tested negative for antibodies to HIV-1 by an enzyme-linked immunosorbent assay (ELISA) (Clavel *et al.*, 1986). Gel electrophoresis and RNA hybridization experiments revealed that this new retrovirus was similar to HIV-1, but was possibly even more closely related to a simian immunodeficiency virus, SIV_{MAC}, the etiological agent of simian AIDS in macaques. Differences between HIV-1 and HIV-2 are highlighted by the fact that natural infection with one HIV type does not prevent infection with the other. Dual infections with HIV-1 and HIV-2 are common on the Ivory Coast, a part of West Africa where both viruses are prevalent (George *et al.*, 1992).

By the end of 1984, South Africa, Rwanda and the Central African Republic had reported cases of AIDS to the World Health Organization. At which time the global number of cases had reached 12,030. This figure doubled in 1985 with cases of AIDS reported to the WHO by 33 American and 20 European, 6 African and 9 Asian/Oceanian countries. By June 1988 this number had climbed to 100,410 reported cases of AIDS in 138 countries, including 29 African nations (Red cross, 1988). AIDS is a truly international disease with incident reports of AIDS from nearly every country. To date the WHO estimates that there are 34 to 46 million cases of HIV infection in the world, 14 million in Africa (UNAIDS and WHO, 2003).

In the USA there have been 1.2 million cases of HIV infection (UNAIDS and WHO, 2003). In 1992 AIDS became the leading cause of death in men aged 25 to 44 years and the fourth leading cause of death in women in the same age group. In 1993 the number of AIDS cases among women increased by 151% and among men by 105% (Quinn, 1995). The incidence of HIV infection in women in the USA is still increasing. Heterosexual transmission accounted for 22% of all new cases of HIV infection diagnosed in 2001/2002 (UNAIDS and WHO, 2002a). Perinatal transmission is decreasing mainly due to maternal use of Zidovudine (UNAIDS and WHO, 2002a). Phylogenetic analysis of regions of the envelope glycoprotein of viruses from infected injecting drug users (IDU) and homosexual men in America and Northern Europe has revealed that HIV-1 found in the European IDU group originated in the USA. The envelope amino

acid sequences of the European IDU viruses clustered with the American IDU viral sequences and not with the viral sequences of European homosexuals (Lukashov *et al.*, 1996).

In the European Union, a union of thirteen countries (Austria, Belgium, Denmark, France, Germany, Greece, Ireland, Italy, The Netherlands, Portugal, Spain, Sweden and the United Kingdom), there is estimated to be a total of 520000 to 680000 HIV infected people (UNAIDS and WHO, 2003), with 30000 – 40000 new cases in 2003 (UNAIDS and WHO, 2004). Among these HIV infected persons three main groups have emerged; 42% were injecting drug users, 25% were homosexual or bisexual men and 18% were heterosexually infected. Estimates of the incidence of AIDS in the European Union suggested an overall annual increase of 24%, with the largest increases in the heterosexually infected group (Downs *et al.*, 1997).

In India a high level of HIV infection was first observed in prostitutes in Goa and in a STD clinic in Bombay where 38.5% of patients were found to be HIV seropositive. In both of these groups, 20% of the infected persons were reactive to both HIV-1 and HIV-2. Genetic analysis of the *env* gene of HIV-1 isolates from both Bombay and Goa showed them to be subtype C and closely related to the South African strain HIV-1_{NOF} (Dietrich *et al.*, 1993). By the end of 2002 between 3.8 and 4.6 million people in India were infected with HIV and in some areas HIV prevalence had reached 1% in pregnant women (UNAIDS and WHO, 2003).

In Thailand, the AIDS epidemic has exploded in two distinct groups; in the injecting drug users seroincidence increased at a rate of 3% to 5% per month with a seroprevalence in 1988 of 32-43% and seroprevalence among prostitutes was 5% in the Thai provinces and 44% in the northern Chiang Mai province (Pau *et al.*, 1993). The pattern of HIV spread throughout Asia is predicted to follow that seen in India and Thailand with heterosexual transmission becoming the major route of infection (Quinn, 1995).

By 2002 the number of people with HIV/AIDS in China was estimated to be 820000. At this early stage of the Chinese epidemic HIV transmission is mainly

in urban areas within the sex worker and injecting drug user populations (UNAIDS and WHO, 2002a and 2003). The World Health Organization estimate that there are already between 4.6 and 8.2 million HIV infected persons in South Asia (UNAIDS and WHO, 2003).

In the mid-1990s the number of reported cases of AIDS in Indonesia was 67, but this was thought to be due to the lack of an HIV surveillance program and the number of infected Indonesians was estimated at around 50000. Genetic analysis of the Indonesian viruses revealed that they were closely related to the Thai viruses of subtypes E and B (Porter *et al.*, 1997). Since 1997 the HIV seroprevalence has increased sharply and by 2001 0.015% of blood donations were HIV positive (UNAIDS and WHO, 2002b). In 2000-2001 HIV seroprevalence of sex workers varied from 0% to 26.5% in different areas of Indonesia and the seroprevalence of IDUs was as high as 53% in Surabaya (UNAIDS and WHO, 2002c).

In Central and South America since 1985 the number of HIV infected persons has risen steadily from about 400000 to nearly 2 million cases: with 610000 cases in Brazil and 150000 cases in Mexico (UNAIDS and WHO, 2002a). In Haiti the national HIV prevalence is 5.6%; 13% in the north west and 2% in the south. The Bahamas and Trinidad and Tobago have an HIV prevalence of 3% (UNAIDS and WHO, 2004b).

In Oceania the incidence is lower than in other areas of the world with 12000 to 18000 AIDS cases (UNAIDS and WHO, 2003). Of these 12000 were in Australia, and by the end of June 2000 719 AIDS cases and 1456 cases of HIV infection had been reported in New Zealand (UNAIDS and WHO, 2002a).

Appendix I shows the number of persons living with HIV infection or AIDS estimated by the World Health Organization at the end of 2003 (UNAIDS and WHO, 2003).

1.2 HIV-1 in sub-Saharan Africa

The emergence of AIDS in sub-Saharan Africa took a different pattern to that seen in America. A wasting disease, known locally as "slim disease", first appeared in East Africa in the Ugandan district of Rakai and since the first reports 19 years ago (Serwadda *et al.*, 1985) the epidemic in Uganda had escalated so that by 1995 at 46,000 Uganda had the highest number of reported AIDS patients of any African country (World Health Organization, 1995b). HIV-1 seroprevalence in adults reached 8.2% in the rural district of Masaka (Wagner *et al.*, 1993) and in the capital city of Kampala, antenatal clinics recorded seroprevalence as high as 30-40% (Mulder, 1995). In south western Uganda, in the Rakai district (See Appendix II - Map of Uganda showing these areas) 8% of the people in agricultural villages were seropositive and in the main road trading centres up to 47% of women were infected with HIV-1 (Wawer *et al.*, 1991). Since the mid-1990s studies carried out in rural south western Uganda have indicated a decline in the overall seroprevalence of HIV-1 (Kamali *et al.*, 2000; Whitworth *et al.*, 2002). In 2000 Kamali *et al.* reported a decline in seroprevalence from 8.2% to 6.9% over 7 years, but no corresponding decline in seroincidence (Kamali *et al.*, 2000). In 2002 seroprevalence had continued to drop to 6.4% (Whitworth *et al.*, 2002). From this rural cohort early indications are that the HIV-1 epidemic in Uganda may have at last reached a plateau. However the earlier pattern of escalating HIV infection in Uganda has been echoed in many sub-Saharan African nations since 1985 as outlined by the following seroprevalence studies.

In 1992 5000 new antenatal patients attending the Langata clinic in Nairobi, Kenya were screened for HIV-1 seroreactivity. HIV-1 seroprevalence was 13% in the group, suggesting a rapidly increasing annual incidence rate of 3 - 4% among pregnant women in Nairobi (Temmerman *et al.*, 1992).

In 1987 HIV-1 and HIV-2 seroprevalence was investigated at a rural hospital in north western Tanzania. In order to avoid any bias 253 consecutive patients were examined and the total HIV seroprevalence was 4.4%. HIV-1 antigen or antibodies to HIV-1 were present in 3.6% of the study group, 0.8% had

antibodies to HIV-2 and 0.4% had antibodies to both. In the nearby town of Bukoba 16% of pregnant women and 13.9% of blood donors were found to be HIV-1 seropositive. Risk assessment was carried out and an association was established between HIV infection and a history of gonorrhoea. Contact with people from Rwanda and Uganda was also associated with an increased likelihood of HIV infection (Schmutzhard *et al.*, 1989). In 1989/90 Shao *et al.* carried out a population-based study of HIV-1 in 4086 subjects in northwest Tanzania. Four study groups were identified and seroprevalence were assessed; in the high risk group seroprevalence was 13%, in the urban group 8.8%, in the peri-urban group 6.5% and in the rural group 2.6% (Shao *et al.*, 1994).

In 1988 500 consecutive patients attending a STD clinic in Addis Ababa were tested for HIV-1 antibodies: 12% of these patients were found to be HIV-1 seropositive. The seroprevalence of this group of patients did not reflect that of the general population of Ethiopia, because they were attending a STD clinic. It did however show that HIV-1 infection may be associated with other STDs and that in 1988 HIV-1 had recently reached Ethiopia (Kefenie *et al.*, 1991). In 1994 13000 AIDS cases had been reported in Ethiopia (World Health Organization, 1995a). A few years later a survey in Addis Ababa estimated that seroprevalence in the general population was between 10% and 27%, and in commercial sex workers was as high as 47% to 59% (Abebe *et al.*, 1997).

The overall trend in the late 1990s across East Africa has been a gradual decline in HIV-1 prevalence which has levelled off at about 8.5% (Asamoah-Odei *et al.*, 2004). This gradual decline may be due to changes in sexual behaviour, better and more widespread treatment of STDs and use of condoms, or simply due to a naturally occurring plateau in the epidemic.

In Central Africa the overall prevalence of HIV-1 has levelled off at around 5%, but it is important to realise that regional seroprevalence rates may differ from this figure (Asamoah-Odei *et al.*, 2004).

HIV-1 seropositivity and mortality were studied in Kinshasa, The Democratic Republic of the Congo in 1987. Seroprevalence in the general population of

Kinshasa were reported at between 3% and 7%, and AIDS accounted for 43% of 500 consecutive adult deaths at the University hospital (Nelson *et al.*, 1991). In 1994 a seroepidemiological study of HIV in the north western region of The Democratic Republic of the Congo revealed HIV-1 seroprevalence at 48.5% and HIV-2 seroprevalence at 4.5% (Bernal *et al.*, 1994).

A study in 1992/93 of 1233 pregnant women attending antenatal clinics in Kigali (Rwanda) revealed that 34% were HIV-1 seropositive (Leroy *et al.*, 1995). During the same period of time in southern Rwanda 1150 women enrolled in a 2 year survey of HIV-1 seroincidence: over the study period HIV-1 incidence was 2.7% overall with a peak incidence of 10.5% in teenagers (Bulterys *et al.*, 1994).

In the Central African Republic HIV-1 is highly divergent and subgroups A, C, D and E have been identified (Murphy *et al.*, 1993). In a house to house study carried out in 1986 the seroprevalence of men was 1.4% and the seroprevalence of women was found to be 5.5%. In 1989 a study of workers at 22 companies in Bangui found that seroprevalence in men was 11% and women 2.2%. These varying results may be partially explained by the different sample groups covered by the two studies: Working men, such as those in the 1989 study, are thought to have more sexual partners than unemployed men. Women who are unemployed, like those in the home-based 1986 study, may increase their income through prostitution. These activities are likely to increase the HIV-1 seroprevalence in these groups (Mathiot *et al.*, 1990).

Sero-surveillance carried out in Gabon in the 1990s showed that seroprevalence in the general population had remained low and stable at 2%. This pattern was also seen in Cameroon and is in contrast to that seen in the other African nations reporting HIV infection. Genetic analysis of the isolates in these two countries has shown there to be 6 different group M subtypes and group O: in Yaoundé, Cameroon less than 10% of HIV-1 infected persons were infected with group O viruses (Zekeng *et al.*, 1994).

This central African pattern of high genetic diversity and low rate of transmission of HIV-1 has yet to be explained (Delaporte *et al.*, 1996).

West African studies of women attending antenatal clinics have indicated that HIV-1 prevalence has remained constant at about 4% in this part of Africa since 1997 (Asamoah-Odei *et al.*, 2004).

In the Gambia, HIV-2 has been found to be more prevalent than HIV-1; in 1991 26% of prostitutes were found to be infected with HIV-2. In the same group 2.1% were seropositive for HIV-1 and 2.5% were dually infected (Pepin *et al.*, 1991).

In a study of over 5000 hospital admissions in Abidjan, the Ivory Coast 66% of deaths were due to AIDS, with an HIV-1 to HIV-2 ratio of 10:1 in these AIDS mortalities (Lucas *et al.*, 1993). This ratio of HIV-1 to HIV-2 perhaps highlights the slower rate of disease progression or the reduced pathologic effects of HIV-2, as suggested by other researchers (Kanki *et al.*, 1994). Kanki *et al.* studied retrovirus prevalence in West Africa since 1985 and concluded that HIV-2 was more common than HIV-1, but less pathogenic. HIV-2 has a slower heterosexual spread than HIV-1, leading to a prevalence plateau at approximately 11% of the population (Kanki *et al.*, 1987, 1994). In a community based study in Guinea Bassau HIV-2 seroprevalence in 100 randomly selected households (649 adults and 680 children) was 9% in 15 to 40 year olds and 20% in those over 40. The survey reported 4 HIV-2 seropositive children but these infections were hospital acquired and there was no evidence of vertical transmission of HIV-2 (Poulsen *et al.*, 1989).

Since the early 1990s the heterosexually transmitted HIV-1 epidemic in Southern Africa increased. In 1996 HIV-1 prevalence in Malawi and Botswana was up to 30% (Abdool Karim and Abdool Karim, 1999). HIV-1 prevalence in women attending antenatal clinics in Blantyre (Malawi) and Lusaka (Zambia) were 16% and 20% respectively (UNAIDS and WHO, 2003).

In South Africa HIV seroprevalence has risen rapidly since 1990 from 0.76% to 22.8% in 1998. Seroincidence peaked in 1997 at 11.9% in young women aged 15 to 30 years old (Abdool Karim and Abdool Karim, 1999). In the 1980s HIV-

1 infection in South Africa led to a predominantly homosexually transmitted disease of the white population. By 1990 this first epidemic was in decline and the second epidemic was underway. In this second, and more widespread epidemic, infection with HIV-1 leads to a heterosexually transmitted disease of black Africans, who make up 77% of the total population and of whom 45% are less than 20 years old (Gilbert and Walker, 2002). In a huge and on going national survey of women attending 400 antenatal clinics over 16500 women have been tested anonymously and the seroprevalence in 2002 was 26% and still increasing. Levels peaked at 37% in KwaZulu Natal where AIDS caused 48% of all adult deaths (Hosegood *et al.*, 2004).

In a general population study carried out involving 2596 male factory workers in Harare, Zimbabwe between March 1993 and March 1995 19.3% were seropositive at enrolment into the cohort. Seroincidence during the study was 3.2%, and peaked at 4.5% in the age group 21-25 years. HIV seroincidence risk factors were investigated, and men reporting sexually transmitted diseases during the follow-up period of the study were more likely to seroconvert. Increased numbers of sexual partners and sex with prostitutes also increased the likelihood of seroconversion (Mbizvo *et al.*, 1996). A study of the prevalence of HIV-1 has been carried out in two rural districts of Zimbabwe, the Rusitu Valley and the slightly less remote Honde Valley (Gregson *et al.*, 1997). A total of 487 pregnant women were tested for HIV-1 infection. The HIV prevalence in the Rusitu Valley was 14% and in the Honde Valley was 24%. Despite the rural remoteness of these two locations, the HIV-1 epidemic seems to have become wide spread throughout this part of Africa (Gregson *et al.*, 1997).

Vertical transmission of HIV-1 occurs from infected mothers to their children during pregnancy and at birth, and transmission from mother to infant during breast-feeding is another important route of HIV infection (Stiehm & Vink, 1991; Kampinga *et al.*, 1997). In South Africa mother to child transmission is estimated at between 30 and 34% of pregnancies of infected mothers (Bobat *et al.*, 1996, Wilkinson *et al.*, 2000). Vertical transmission of HIV-1 in Uganda was recorded

at 26% from two study groups (Mugerwa *et al.*, 1996). In a study carried out in Addis Ababa, Ethiopia of 92 pregnant mothers infected with HIV-1 and their infants over a 2 year follow up period 68% of the children became infected by the time they were 18 months old (Chamiso, 1996).

However in sub-Saharan Africa heterosexual intercourse represents the main mode of transmission of HIV-1 (Latif *et al.*, 1989; Bernal *et al.*, 1994; Gilbert and Walker, 2002). Heterosexual transmission of HIV-1 is exacerbated by the presence of other sexually transmitted diseases (Hudson *et al.*, 1988; Latif *et al.*, 1989; Schmutzhard *et al.*, 1989). Over the last 15 years HIV-1 has emerged as a major cause of adult death in many areas of Africa including Rwanda, the Ivory Coast, Uganda, The Democratic Republic of the Congo and KwaZulu Natal (De Cock *et al.*, 1990; Nelson *et al.*, 1991; Gregson *et al.*, 1994; Mulder *et al.*, 1994; Hosegood *et al.*, 2004).

Appendix III shows the HIV-1 prevalence among women attending antenatal clinics in Africa and was compiled from surveys carried out between 2001 and 2002 (UNAIDS and WHO, 2002d).

1.3 Clinical

HIV infection almost inevitably leads to AIDS, which for the purpose of accurate disease surveillance has been described in detail by the WHO and the American Center for Disease Control (CDC). A clinical case definition was established in 1985 with a proviso that this might in time need to be adapted to suit disease in different countries (World Health Organization, 1986). AIDS in adults was defined by the presence of two of the major signs and at least one of the minor signs as detailed below:

Major signs:

i/ 10% or greater loss of body weight.

ii/ chronic diarrhoea (longer than 1 month).

iii/ prolonged fever (longer than 1 month).

Minor signs:

i/ persistent cough (longer than 1 month).

ii/ generalized pruritic dermatitis.

iii/ recurrent herpes zoster.

iv/ oral candidiasis.

v/ progressive herpes simplex.

vi/ generalized lymphadenopathy.

Kaposi's sarcoma or cryptococcal meningitis alone also are definitive for AIDS diagnosis. A slightly different definition was detailed for paediatric AIDS.

This clinical case definition of AIDS was revised by the CDC in 1987 (Centers for Disease Control, 1987). In this revision of the clinical case definition in the absence of laboratory evidence for HIV infection more diseases indicative of AIDS were added; these included cryptosporidiosis, cytomegalovirus, primary brain lymphoma, *Pneumocystis carinii* pneumonia, *Mycobacterium* disseminated disease, multifocal leukoencephalopathy and toxoplasmosis of the brain. A detailed list of infections with confirmed HIV infection was also listed in the CDC updated case definition. A further revision of the AIDS case definition was made by the CDC in 1991; when pulmonary tuberculosis, invasive cervical cancer and recurrent pneumonia were added to the list of indicative diseases.

Both the original and the CDC's revisions of the AIDS case definition were appropriate for the identification of AIDS in the USA and other developed countries, but were not consistent in the developing countries where the facilities for diagnosis of the AIDS indicator infections might not be available. Clinical presentation of AIDS in Africa differs from that seen in developed countries this is mainly due to the different microbial environment in which the HIV positive persons are living. The opportunistic infections in tropical locations are associated with the common pathogens present (Biggar, 1986). Enteropathic AIDS, also known as "slim disease", is common in many African countries and was investigated by Sewankambo in Uganda. The symptoms of this disease are substantial weight loss and chronic diarrhoea: the causative agents of the diarrhoea have been identified as cryptosporidiosis and isosporiasis (Sewankambo *et al.*, 1987).

To enable the clinical case definition to be used in developing countries various revisions have been made in different continents and countries. These revisions are weighted towards the predominating local infections that are indicative of AIDS and that can be readily identified with perhaps limited medical facilities. The Caracas case definition used in South America incorporates a point system which weights certain diseases and requires HIV seropositivity for AIDS definition (Buehler *et al.*, 1993).

In Uganda the WHO clinical case definition for Africa was introduced in 1987 and there were educational meetings to teach the district medical officers how to apply the definition (Berkley *et al.*, 1989). Modifications have been made to this African case definition to increase its efficiency in Uganda. A TB cough was excluded from the minor signs and amenorrhea was added (Widy-Wirski *et al.*, 1988).

1.4 Viral Pathogenesis

1.4.1 Correlates of Disease Progression

The assessment of an infected person's disease status and the prediction of when and by how much it will alter is of considerable importance in patient management and therapy. This is becoming even more important with the development and availability of more potent antiviral drugs (Coffin, 1996). One of the main characteristics of AIDS is the severe depletion of the CD4 cells in the blood and measurements of total CD4 cells and CD4:CD8 cell ratios are widely used to monitor patients' health or disease status. CD4+ cell counts were found to give a reliable prognosis of disease in a very comprehensive international study (Multicohort Analysis Project Workshop, 1994). However, it has been shown that plasma viral load is the single best predictor of time of progression to AIDS (Hammer, 1996; Mellors *et al.*, 1996; Saag *et al.*, 1996). Mellors *et al.* measured viral RNA concentrations in plasma in a study of 180 patients over 10 years. The concentrations of subjects' plasma viral RNA were measured on entry into the study; these baseline values varied from <500 to 294000

molecules/ml. The predictive value was significantly better than CD4+ counts, for which only the lowest quartile (<320 cells/μl) gave a significant correlation with disease progression.

The measurements of T cell subsets and viral load are relatively expensive and require specialist facilities and expertise normally available only in developed countries. However, data is now being obtained from other countries including some in Africa. For example, T cell subset analysis is routinely done in Entebbe on blood samples taken from members of the Natural History cohort in rural SW Uganda and, in a 3 month period in 1996, all the HIV-positive samples taken from this cohort were assayed for viral load by Professor F Gotch and her colleagues at the Chelsea & Westminster Hospital, London (Kengeya-Kayondo *et al.*, 1997). These data should eventually allow a detailed comparison of the rates of disease progression in infected Africans and persons in developed countries.

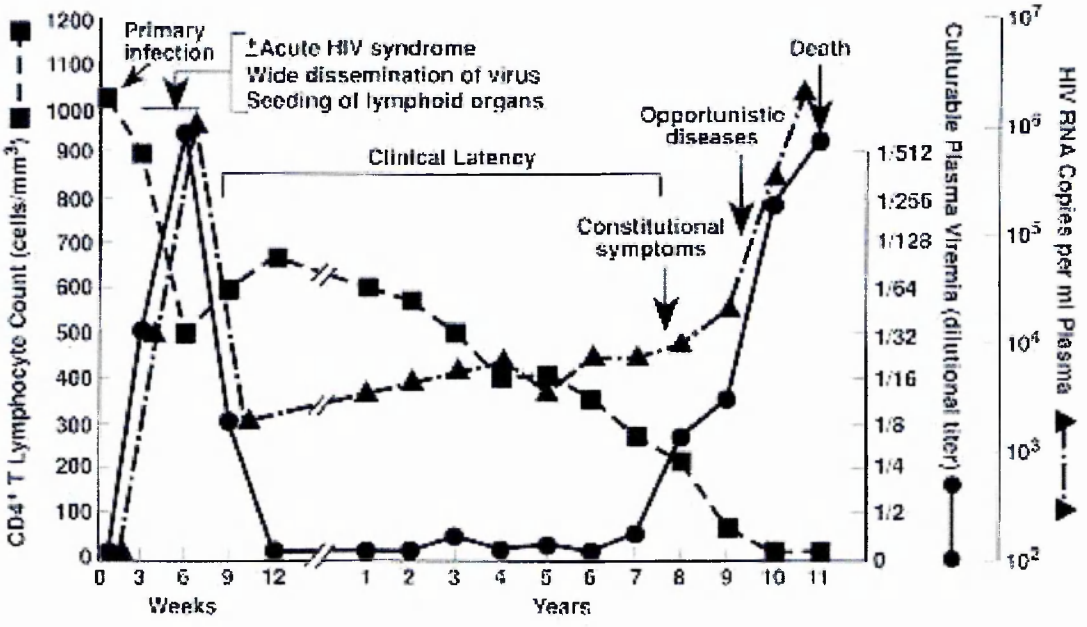
1.4.2 Disease Progression

In most cases, infection with HIV causes a mild, often subclinical, primary illness followed by an asymptomatic phase, which may last for many years, leading eventually to severe immunosuppression, the onset of AIDS and death. This sequence is illustrated diagrammatically in Figure 1 (Pantaleo, 1993).

The acute phase is characterized by a high viral load (illustrated in Figure 1 by the RNA copies per ml of plasma) in the blood which triggers humoral and cell mediated immune responses to antigenic epitopes in viral proteins. These responses then lead to a large reduction in the plasma viral load, which may be maintained at a low level throughout an asymptomatic phase which can last for many years. However, the development of severe immunodepression, associated with the destruction of many of the plasma lymphocytes (illustrated in Figure 1 by the CD4+ T lymphocyte count), leads to a large increase in viral load, opportunistic infections and death.

Figure 1. Schematic representation of the time course of disease progression from HIV infection to AIDS and death. It illustrates the changes in plasma viral load and the CD4+ lymphocyte level/ml of blood at various stages (Figure adapted from Pantaleo, 1993).

Figure 1


Primary infection
Acute HIV syndrome
Wide dissemination of virus
Seeding of lymphoid organs
Clinical Latency
Opportunistic diseases
Constitutional symptoms
Death
CD4+ T Lymphocyte Count (cells/mm³)
Culturable Plasma Viremia (dilutional titer)
HIV RNA Copies per ml Plasma
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
1:512
1:256
1:128
1:64
1:32
1:16
1:8
1:4
1:2
0
10⁷
10⁶
10⁵
10⁴
10³
10²
0 3 6 9 12 1 2 3 4 5 6 7 8 9 10 11
Weeks
Years

It used to be thought that the low levels of plasma virus following the clearance of virus by the primary immune response reflected a very low viral turnover during the asymptomatic stage, which could almost be regarded as a latent phase. However, studies have shown that the rate of turnover of virus and virus infected cells is much greater than previously believed: quantitative data obtained in the mid-1990s led to a marked change in our understanding of viral pathogenesis (Ho *et al.*, 1995; Wei *et al.*, 1995). These two groups studied the changes in plasma viral load and CD4+ T cell turnover in peripheral blood of a total of 42 asymptomatic persons with CD4+ T cell counts ranging from 18 to 480 per mm^3 and plasma viraemia ranging from 15 to 554 virions /ml $X10^3$. The mean half-life for plasma virus turnover was determined at 2 days, and the mean doubling time for infected CD4+ T cells in peripheral blood was found to be 15 days. The average rate of CD4+ T cell turnover in peripheral blood was calculated at $4 X 10^5$ cells per day, and the average total HIV-1 production was $6.8 X 10^8$ virions per day (Ho *et al.*, 1995; Wei *et al.*, 1995). They concluded from these data that there is a continuous turnover of virus infected cells during the asymptomatic phase of infection and that during this phase the rate at which infected PBMC are destroyed is balanced by the rate of cell replacement, so for a time the plasma viral load and the number of CD4+ T cells are in dynamic equilibrium.

Wolthers *et al.* (1996) assessed the rate of CD4+ and CD8+ T cell turnover in samples from 14 HIV-1 infected persons from the early asymptomatic stages and up to the diagnosis of AIDS using the indirect method of telomere measurement. They observed no decrease in CD4+ T cell telomere length and concluded that CD4+ T cell turnover in HIV infected persons was no higher than that of uninfected persons. This study also noted an increased CD8+ T cell turnover, suggesting that this cell type was infected, but was replenished during HIV infection at an elevated rate (Wolthers *et al.*, 1996). In contrast to the "Sink analogy" made by Ho *et al.*, Wolthers *et al.* suggested that the CD4+ T cell depletion was due to the effect of HIV-1 infection on CD4+ T cell precursors,

which in some way hindered the replacement of CD4+ T cells (Wolthers *et al.*, 1996).

Further studies by Mohri *et al.* (2001) and Ribeiro *et al.* (2002) supported the model for T cell dynamics proposed by Ho *et al.* (1995) and Wei *et al.* (1996). Using a deuterium labelling system they investigated the *in vivo* T cell turnover dynamics of 7 asymptomatic HIV infected persons with CD4 + T cell counts ranging from 48 to 804 cells per mm^3 and plasma viral loads from 7.6×10^3 to 5.0×10^5 RNA copies per ml. They compared their T cell dynamics with those of 4 aged matched healthy controls (Mohri *et al.*, 2001; Ribeiro *et al.*, 2002). They showed that in the HIV-1 infected persons the CD4+ T cell activation rate was increased, whereas the CD8+ T cell activation rate was not. The CD4+ T cell death rate was increased, but the CD8+ T cell death rate was not. The fraction of activated T cells was significantly increased in the CD8+ T cell population, because the activated CD8+ T cells remained active and proliferated for a longer time before recycling to a resting state (Mohri *et al.*, 2001; Ribeiro *et al.*, 2002).

Other researchers have found an increased turnover of CD4+ and CD8+ T cells in HIV-1 infected persons. Sachsenberg *et al.* (1998) found the mean daily turnover of CD4+ and CD8+ T cells was 2-times and 6-times higher respectively, and that the CD8+ CD45RO+ T cell subset was selectively expanded (Sachsenberg *et al.*, 1998). Lempicki *et al.* (2000) also found increased CD4+ and CD8+ T cell turnover: comparing 57 HIV-1 infected persons with 67 healthy controls. They found that the HIV infected persons had increased fraction of proliferating CD4+ and CD8+ T cells (Lempicki *et al.*, 2000).

Hellerstein *et al.* (2003) used a short-term and a long-term deuterium labelling system to establish that the human T cell memory / effector cell sub-population is made up of short-lived and long-lived cells. They found that in response to advanced HIV-1 infection the number of proliferating CD4+ and CD8+ T cells increased, and the ratio of short-lived (effector) cells to long-lived (memory) cells increased. In uninfected controls the short-lived cells made up 4 to 6% of the CD4+ T cell pool and in persons with advanced HIV-1 infection this had increased to 20 to 25%. They concluded that HIV-1 infection alters the

development of T cells towards an increased fraction of short-lived cells (Hellerstein *et al.*, 2003).

Viral titres do not vary significantly within a patient during the asymptomatic phase (Mellors *et al.*, 1996) indicating that a dynamic equilibrium is established after seroconversion so that the viral load remains relatively low and stable during the asymptomatic phase. Eventually, however, the equilibrium becomes unbalanced. The virus load in the peripheral blood, which is indicative of the total replicating virus in the various lymphoid tissues, becomes high enough to outstrip the rate at which the infected CD4+ T cells destroyed by virus or by the immune system can be replaced. At this stage the immune system breaks down and the opportunistic infections characteristic of AIDS develop (Bednarik & Folks, 1992; Coffin, 1996; Saag *et al.*, 1996).

It has become apparent from studies in several developed countries that the rate of disease progression varies between HIV-infected persons, who have been divided into three groups; rapid progressors, typical progressors and nonprogressors. Rapid progressors may develop AIDS 2 to 3 years after HIV infection (Phair *et al.*, 1992). Their antibody response to viral proteins is lower than the responses seen in the other two groups, as is the ability of their antibodies to neutralize autologous virus (Pantaleo *et al.*, 1995; Haynes *et al.*, 1996). The viral load following initial HIV infection remains high, suggesting an inefficient immune response (Mellors *et al.*, 1995). Typical progressors remain asymptomatic for 8 years after HIV infection (Hendriks *et al.*, 1993; Hendriks *et al.*, 1998). Their viral load drops following the primary immune response to HIV infection (Mellors *et al.*, 1995) which includes neutralizing antibodies and cytotoxic T cells to immunodominant HIV epitopes (Arendrup *et al.*, 1992; Koup *et al.*, 1994). Nonprogressors may be HIV positive for 15 years or longer without developing AIDS (Sheppard *et al.*, 1993; Pantaleo *et al.*, 1995). Nonprogressors have a high level of neutralizing antibodies and a persistent high level of CTL in

their peripheral blood, especially CD8+ CD38- CTL (Haynes *et al.*, 1996; Rosenburg *et al.*, 1997).

Compared with the rate in HIV-infected Americans and Europeans as outlined above (Phair *et al.*, 1992; Hendriks *et al.*, 1993; Sheppard *et al.*, 1993; Hendriks *et al.*, 1998), the rate of disease progression appears to be faster in many infected persons in African. In one study 163 HIV-1-infected female Kenyan sex workers were followed from seroconversion to the time of HIV-related disease, AIDS and death. The mean time from seroconversion to the first onset of clinical symptoms was 3.5 years, and the time from seroconversion to progression to AIDS was 4.4 years (Anzala *et al.*, 1995). In a study of HIV-infected Africans attending the clinics in the Natural History cohort in rural SW Uganda the time from seroconversion to progression to AIDS was 4.4 years, and the time from seroconversion to death was less than 6 years (Morgan *et al.*, 1997).

It has been postulated that more rapid disease progression in some infected persons in African is due to higher rate of exposure to pathogens which leads to frequent or chronic activation of virus-susceptible cells (Bentwich *et al.*, 2000). Helminthic infections are common in many parts of Africa and may be involved in immune activation of T helper 2 cells which is thought to facilitate the progression to AIDS in HIV-1 infected persons (Bentwich *et al.*, 1995). In many parts of sub-Saharan Africa the incidence of *Mycobacterium tuberculosis* more than 25% (Bundy *et al.*, 2000). *Mycobacterium tuberculosis* infection can result in the increased expression of the HIV co-receptor CCR5 (Fraziano *et al.*, 1999) and is associated with a higher rate of HIV-1 replication (Goletti *et al.*, 1996; Garrait *et al.*, 1997).

Clearly, the factors involved in different rates of progression are not fully understood and it seems probable that the rate depends on host as well as viral factors. For example, it has been proposed that some rapid progressors do not mount an effective CTL response (Wolinsky *et al.*, 1996). Others have proposed that differences in progression rate correlate with HLA halotype (Steel *et al.*, 1988). Furthermore, African HLA halotypes are different to those in Caucasians

and genetic factors may also affect the rate of progression in Africans. The homozygous deletion in CCR5 gene, which results in the production of a non-functional receptor for HIV appears to be confined to Caucasians and would not confer protection to Africans (Dean *et al.*, 1996; Samson *et al.*, 1996).

1.4.3 Virus / Host Interactions during Disease Progression

It is now apparent that different types of cells and tissues may be important sites of HIV-1 replication at different phases of disease progression and viral variants isolated at different phases may show different cellular tropisms.

At the time of sero-conversion the main sites of virus replication are CD4-positive cells in the blood (macrophages, T4 cells and dendrocytes) but at later on other tissues and organs may be infected, including the brain (Bottiger *et al.*, 1991; Epstein *et al.*, 1991), thymus (Beaulieu *et al.*, 1996), lungs (Agostini *et al.*, 1995) and gut (Donaldson *et al.*, 1994).

Livingstone *et al.* measured the proviral loads in cell fractions from HIV-infected patients and showed that CD8 cells, together with dendrocytes, may form a major site for HIV replication in many patients (Livingstone *et al.*, 1996). However, fewer patients had significant amounts of HIV provirus in their monocytes. There was a significant reduction in the number of circulating CD8 lymphocytes in patients with low CD4 counts. The mechanism of infection of CD8 lymphocytes, which as mature cells do not express CD4, is uncertain but the authors proposed that it could be via infection of immature lymphocytes in the thymus or by a CD4-independent mechanism involving chemokine receptor molecules (see Section 1.5.4).

Two groups of primary isolates have been defined, depending on their ability to infect macrophages or infect and induce the formation of syncytia in immortalized CD4+ cell lines (Åsjö *et al.*, 1986; Fenyö *et al.*, 1988; Cheng-Mayer *et al.*, 1989; Tersmette *et al.*, 1989). The majority of primary HIV-1 isolates replicate to low levels in peripheral blood mononuclear cells (PBMC) and do not replicate

in immortalized T cell lines (Åsjö *et al.*, 1986; Schuitemaker *et al.*, 1991; De-Jong *et al.*, 1992; Connor *et al.*, 1993; Connor & Ho, 1994). These isolates are classified as macrophage-tropic (M-tropic) viruses. Some M-tropic viruses are also able to replicate in primary T cells; some of these induce syncytia in primary T cells (SI viruses), but others (NSI viruses) do not. At late stages in infection viruses may appear which can infect and induce the formation of syncytia in immortalized CD4+ cell lines (Åsjö *et al.*, 1986; Schuitemaker *et al.*, 1991; De-Jong *et al.*, 1992). These viruses are classified as T cell line-tropic (T-tropic) primary viruses, some of which have yielded laboratory adapted viruses which have lost the ability to replicate in primary macrophages (Schuitemaker *et al.*, 1991; Westervelt *et al.*, 1992; Keys *et al.*, 1993). Almost all viruses isolated shortly after sero-conversion are M-tropic and NSI (De-Jong *et al.*, 1992; Zhang *et al.*, 1993; Zhu *et al.*, 1993; Fiore *et al.*, 1994) and appear to be responsible for sexual transmission (Zhu *et al.*, 1993). T-tropic SI variants emerge later and are often associated with the development of AIDS (Tersmette *et al.*, 1989; Connor *et al.*, 1993).

The differences between M- and T-tropic viruses may have been over-emphasized as Valentin *et al.* found that all the primary isolates they tested, including rapid/high SI and slow/low NSI viruses, were able to infect macrophages (Valentin *et al.*, 1994). Also, Simmons *et al.* found that many primary isolates, both NSI and SI, some which had been biologically cloned, and some T cell line adapted viruses were able to replicate in macrophages to give virus titres similar to those obtained from PBMC cultures (Simmons *et al.*, 1996).

The role of macrophage infection during AIDS has been highlighted by the observation (Orenstein *et al.*, 1997) that large amounts of HIV-1 are produced by these cells when the CD4+ cell count is low. Previously, the source of the increased viraemia at this stage of disease had been unexplained.

The presence of a persistent latent reservoir of HIV-1 infection in resting CD4+ T cells has been demonstrated *in vivo* (Chun *et al.*, 1997; Kinter *et al.*, 2003; Siliciano *et al.*, 2003). Viral DNA has been detected in two forms in resting CD4+ T cells; extrachromosomal and integrated into the cell genome (Zack *et al.*, 1990; Spina *et al.*, 1995; Finzi *et al.*, 1997; Hermankova *et al.*, 2003).

Zack *et al.* (1990) demonstrated *in vitro* that HIV-1 is able to enter these inactive cells and although viral RNA is partially reverse transcribed, integration of the DNA rarely occurs. It is possible to produce progeny virus from resting CD4+ T cells if mitogenic stimulation occurs within 2 days of infection, after this time unintegrated viral DNA is broken down. Such breakdown of viral DNA may account for the large number of apparently uninfected CD4+ T cells in circulation (Zack *et al.*, 1990). Spina *et al.* showed that resting CD4+ T cells could be infected by HIV-1 *in vitro.* After 3 to 5 days they detected stable, full length extrachromasomal viral DNA. They showed low levels of early mRNA transcription and demonstrated that the addition of IL-2 and PHA would induce the production of infectious virus (Spina *et al.*, 1995).

In contrast Finzi *et al.* found that resting CD4+ T cells from asymptomatic HIV-1 infected subjects harboured integrated viral DNA. This study isolated replication-competent virus from 0.2 to 16.2 per 10^6 purified resting CD4+ T cells of asymptomatic patients undergoing antiviral therapy (Finzi *et al.*, 1997).

Hermankova *et al.* (2003) demonstrated that HIV-1 DNA in the resting CD4+ T cells of patients receiving HAART was predominantly integrated. This group found 100 HIV-1 DNA molecules / 10^6 resting CD4+ T cells. They found that only 1% of these infected resting cells could be induced to produce mRNA at high levels and that a subset of these could also produce infectious virus (Hermankova *et al.*, 2003).

Chun *et al.* (1998) examined the resting CD4+ T cells of 10 HIV-1^+ subjects 10 days to 4 months after primary infection for a duration of 0.2 to 17 months after the initiation of HAART. The resting CD4+ T cells contained integrated viral DNA, which yielded infectious virus after cell activation in all 10 cases. They speculated that the reservoir of latently infected CD4+ T cells could have become established in early acute HIV-1 infection when plasma viral load is high and the level of CD4+ T cell activation is elevated (Chun *et al.*, 1998).

1.5 Virology

1.5.1 Classification

HIV is a member of the lentivirus group of the *Retroviridae* family of viruses which have been found in a wide variety of mammals (Coffin, 1991). Other members of the lentivirus group include Visna / Maedi virus and Caprine arthritis-encephalitis virus found in sheep and goats (Murphy *et al.*, 1995), equine infectious anaemia virus found in horses (Cheevers and McGuire, 1985), bovine immunodeficiency virus of cattle (Snider *et al.*, 2003) and the feline lentiviruses found in lions, cheetahs, pumas and bobcats and which are closely related to the feline immunodeficiency virus found world wide in domestic cats (Brown *et al.*, 1994; Olmsted *et al.*, 1992). Another member of the group is simian immunodeficiency virus (SIV) which infects as many as 33 non-human primates is sub-Saharan Africa and captive Asian macaques (Peeters *et al.*, 2002). The primate lentiviruses HIV and SIV may be grouped by phylogenic analysis into 7 major lineages (Courgnaud *et al.*, 2003). HIV-1 and SIV_{CPZ} which infects chimpanzees are grouped together in one of these 7 lineages; and within this lineage the 3 HIV-1 subgroups M, O and N are represented (Gao *et al.*, 1999). HIV-2 is grouped together with SIV_{SM} and SIV_{MAC} from sooty mangabeys and captive macaques respectively (Sharp *et al.*, 1995). The discovery that macaque monkeys develop AIDS when infected with SIV and the similarities between HIV and SIV has led to the use of macaques as an animal model for HIV infection of man (This concept is discussed in section 1.8.2). The five other primate lentivirus lineages are represented by: SIV_{AGM} isolated from various species of African green monkey, SIV_{SYK} isolated from Sykes' monkeys, SIV_{LHOEST} isolated from l'Hoest monkeys (which is grouped together with SIV_{MND1} from mandrills and SIV_{SUN} from sun-tailed monkeys), SIV_{COL} isolated from guereza colobus and SIV_{GSN} isolated from greater spot-nosed monkeys (Courgnaud *et al.*, 2003).

1.5.2 Viral Structure

The HIV-1 virion is a spherical structure approximately 100nm in diameter surrounded by a lipid bilayer. Figure 2 shows the virion with the component parts of the virus labelled (Nye & Parkin, 1994). The virion core is contained by the p24 capsid protein, which encases the two strands of viral RNA, the p9 nucleocapsid protein and the enzymes protease, reverse transcriptase (RT) and integrase. The virus envelope consists of an outer lipid bilayer through which the gp41 transmembrane glycoproteins extend and attach to the gp120 envelope glycoproteins and within, it contains an inner layer of p17 matrix protein (Nye & Parkin, 1994).

The Envelope Glycoprotein

Approximately 70 - 80 envelope glycoprotein molecules are located on the virus surface. Each glycoprotein is synthesised as a gp160 precursor molecule which is enzymically cleaved to form the gp120 and the non-covalently attached gp41 transmembrane molecules. The gp120 molecule consists of a 60kd polypeptide backbone within which there are 24 asparagine-linked glycosylation sites; the positions of 13 of the glycosylation sites are conserved, but 11 of them are located in hypervariable regions and their positions may vary in different isolates. There may be as much as 30% variation in the amino acid sequence of the gp120, this variation is greatest in the hypervariable regions of the glycoprotein, shown in figure 3 as V1, V2, V3, V4 and V5. Figure 3 (Leonard *et al.*, 1990) represents the structure of a gp120 molecule of the HIV-1_{IIIB} isolate. There are 5 disulphide bonded loop structures in the gp120 molecule: the fourth loop is a simple, single disulphide loop which contains the third hypervariable (V3) region. The binding site for the CD4 cell receptor for HIV has been mapped to the second, third and fourth conserved domains of the gp120, shown in figure 3 as C2, C3 and C4. The CD4 binding site is thought to have linear epitopes in the closely associated C3 and C4 hydrophilic domains located near to the third and fifth hypervariable regions of the gp120 and these may be conformationally affected by amino acid residues in the C2 and C4 hydrophobic domains

Figure 2. Structure of an HIV virion with the component parts of the virus labelled. The conical nucleocapsid is encased in a lipid bilayer envelope in which are embedded the surface and transmembrane glycoprotein molecules. The whole structure is a sphere of approximately 100nm in diameter (Nye & Parkin, 1994).

Figure 2:

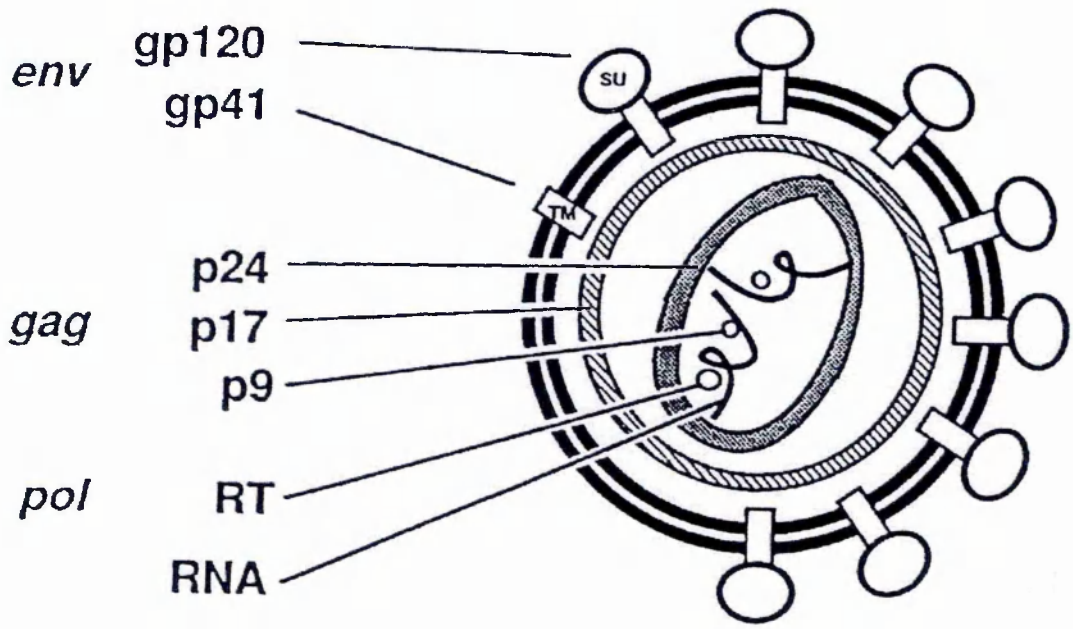
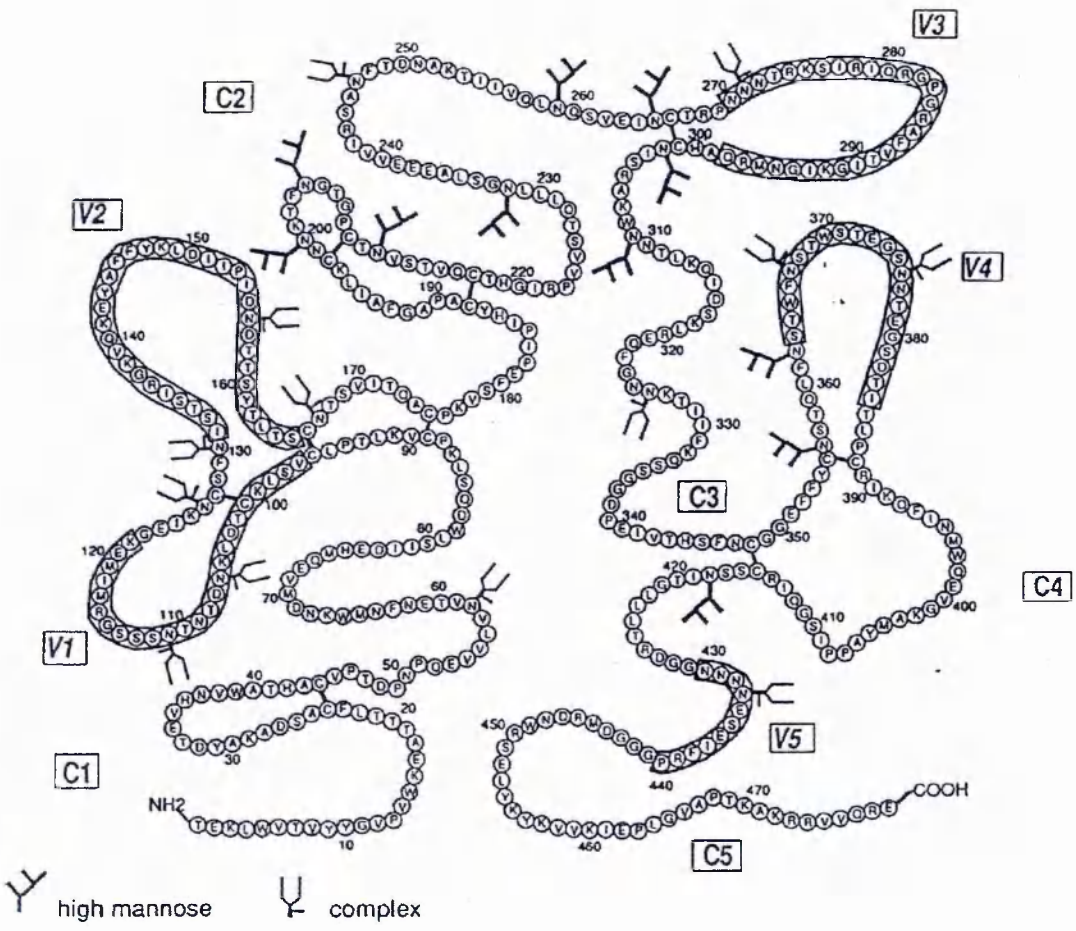


Figure 3. Schematic representation of the surface glycoprotein molecule (gp120) of HIV-1_{IIIB}. The amino acids in the peptide backbone are numbered and the carbohydrate branches and disulphide bonds are identified. The positions of the conserved regions (C1-C5) and the hypervariable regions (V1-V5) are indicated (Leonard *et al.*, 1990).

Figure 3:



(Olshevsky, 1990). As suggested by the location of the CD4 binding site in a conserved part of the gp120 structure, this epitope is conserved across many subtypes of HIV-1 and maybe of importance in the development of a vaccine or for an antiviral treatment post-infection (Pinter *et al.*, 1993; Parren *et al.*, 1995).

The V3 loop has a relatively conserved structure consisting of a loop of 30 to 35 amino acids with an overall positive charge. This loop held together at its base by a disulphide bond and with a type II ...-turn at the apex (LaRosa *et al.*, 1991; Kwong *et al.*, 2000). There is much experimental evidence that the V3 loop region is important in the interactions between the virus and host cells and the immune system. For example, sequence analysis has revealed that amino acid variations in the areas flanking the apex of the loop correlate with the differences in viral phenotype and cell tropisms. Syncytium-inducing isolates have positively charged amino acids in positions 11 and 28 of the V3 loop, and non-syncytium-inducing isolates have neutral or negatively charged residues in these positions (Chesebro *et al.*, 1992; Fouchier *et al.*, 1992; Cao *et al.*, 1993).

Amino acids in the V1, V2, C2, V3 and CD4-binding regions of the gp120 molecule have been shown to be involved with the tropism of an isolate. The apex of the V3 loop region appears to be especially important in determining whether a virus is M- or T-tropic (Hwang *et al.*, 1991; Cann *et al.*, 1992; De-Jong *et al.*, 1992). Further, viral tropism can be changed by point mutations in the *env* gene that cause a single amino acid substitution in the V1 loop apex (Boyd *et al.*, 1993). The growth of HIV-1 chimeras in microglial cells cultured *in vitro* was found to depend on the V3 loop sequence and could be blocked by V3-specific antisera, indicating that this region also determines viral tropism for brain cells (Sharpless *et al.*, 1992).

The V3 loop appears to be of major importance in the attachment of the virus to receptor molecules at the surface of susceptible cells. Several groups have shown that the amino acid sequence of the V3 loop is important in second or co-

receptor usage (see below), determining the specificity of co-receptor binding (Choe *et al.*, 1996; Cocchi *et al.*, 1996; Kozak *et al.*, 1997). Hence, it seems probable that the V3 loop may form part of the region of gp120 which binds to the secondary receptor.

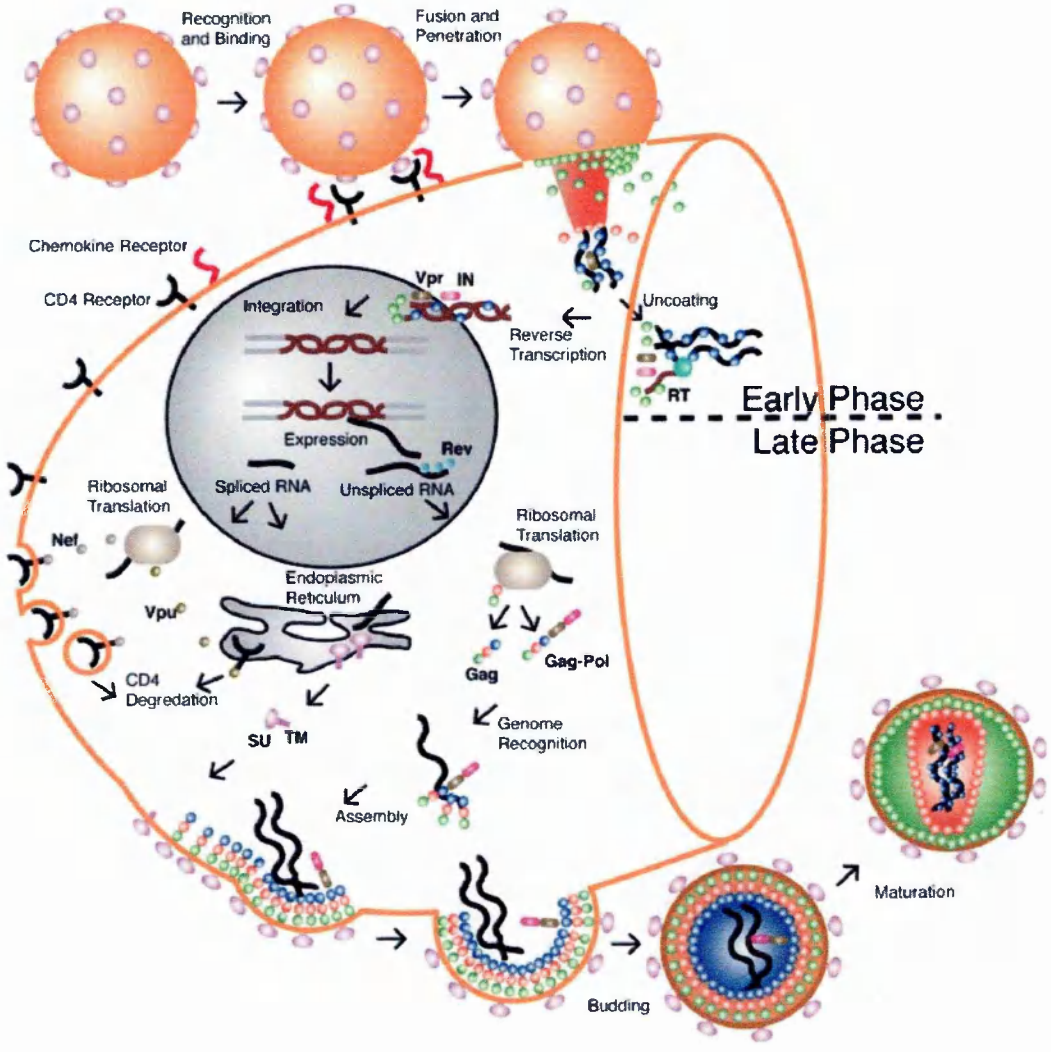
The V3 loop is also a major immunological determinant, inducing both humoral and cell mediated immunity to the virus. The presence of a B-cell epitope, which has been described as the principal neutralizing determinant (PND), is evidence of the immunological importance of this region (Goudsmit *et al.*, 1988; Matsushita *et al.*, 1988; Palker *et al.*, 1988; Rusche *et al.*, 1988; Javaherian *et al.*, 1989; Kenealy *et al.*, 1989). The peptide sequence around the loop apex acts as an epitope which induces specific antibodies in most infected and vaccinated persons and these can be analyzed in peptide-based enzyme immuno assays, which were reviewed by Moore (1993). Small peptides encoded by the V3 loop have been shown to induce neutralizing antibodies in experimental animals (Palker *et al.*, 1988; Javaherian *et al.*, 1989, 1990; Durda *et al.*, 1990; Neurath *et al.*, 1990; Jiang *et al.*, 1992) and although some early studies indicated that neutralizing antibodies were isolate-specific, later data indicates that peptides which include the conserved GPGR loop apex induce neutralizing polyclonal or monoclonal antibodies with a broader specificity (Javaherian *et al.*, 1990; Gorny *et al.*, 1992). In addition, the loop apex is a target for cytotoxic T cells in mice immunized with recombinant HIV gp160 or a consensus V3 peptide (Takahashi *et al.*, 1986; Jiang *et al.*, 1992).

1.5.3 Virus Replication

Early work was focused on the infection and growth of HIV in cell lines derived from the T helper sub-set of lymphocytes which express the CD4 glycoprotein on the surface. Most of our detailed knowledge of the replicative cycle has been acquired from studies with infected CD4+ lymphocytes, and is described below and illustrated in figure 4 (Turner and Summers, 1999).

Figure 4. Schematic representation of the virus replication cycle in an infected CD4+ lymphocyte. The replication cycle commences with the virus binding to receptor molecules at the cell surface followed by fusion of the viral and host cell membranes and ends with the release of progeny virus which bud through the host cell membrane and mature (Turner and Summers, 1999).

Figure 4


Recognition and Binding
Fusion and Penetration
Chemokine Receptor
CD4 Receptor
Integration
Vpr IN
Reverse Transcription
Uncoating
RT
Early Phase
Late Phase
Expression
Rev
Spliced RNA
Unspliced RNA
Ribosomal Translation
Nef
Vpu
Endoplasmic Reticulum
CD4 Degredation
SU TM
Ribosomal Translation
Gag
Gag-Pol
Genome Recognition
Assembly
Budding
Maturation

Binding to the CD4 receptor molecule on the cell surface induces conformational changes in the gp120 which is followed by fusion of the virus envelope with the cell membrane; this is a poorly understood stage in the replication cycle which involves a fusogenic domain in the gp41 transmembrane glycoprotein and cell surface receptors CXCR4 and CCR5 (as discussed in section 1.5.4) (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Choe *et al.*, 1996; Feng *et al.*, 1996; Clapham *et al.*, 1991). Once inside the cell the viral enzyme reverse transcriptase (RT) transcribes the viral RNA into double stranded DNA (Whitcomb and Hughes, 1992). The dsDNA migrates towards the cell nucleus as a preintegration complex, where the Vpr accessory protein connects it to the cellular nuclear import system (Miller *et al.*, 1997; Nie *et al.*, 1998). Vpr also stops the host cell cycle in the G2 phase (Jowett *et al.*, 1995). Inside the nucleus viral integrase integrates the DNA into the cellular chromosomal DNA (Vink and Plasterk, 1993). Following viral genomic integration, viral gene expression is controlled by both cellular and viral factors. Cellular activation leads to the early expression of regulatory and accessory viral genes; *tat, rev* and *nef,* and to the late expression of accessory genes *vif, vpr* and *vpu* and the structural genes *gag, pol* and *env* (Kim *et al.*, 1989; Felber *et al.*, 1990). HIV mRNA is produced and migrates from the nucleus to the host cell ribosomes, where translation occurs. Early gene expression produces Tat mRNAs that are fully spliced. Tat is a transcriptional trans-activator of HIV genes, which binds to the transactivation response element (TAR) at the 5' end of HIV RNAs and stimulates transcriptional elongation leading to the production of full-length viral mRNA molecules in the cell nucleus (Feinberg *et al.*, 1991). The rev protein regulates the splicing of mRNA transcripts in the nucleus and their export to the cytoplasm prior to translation (Emerman and Malim, 1998). Nef is a phosphoprotein that down regulates the surface expression of CD4 (Garcia and Miller, 1992), and has also been seen to enhance HIV infection of primary lymphocytes and macrophages (Balliet *et al.*, 1994; Miller *et al.*, 1994). The *vif* gene product is a 23kd protein which enhances virus infectivity of cells by species-specific interaction with a cellular factor (Simon *et al.*, 1998). The vpu protein is involved

in the dissociation of gp160 from CD4 receptors in the endoplasmic reticulum in the production of the gp120 and gp41 in viral protein assembly (Schnittman & Fauci, 1994) and enhances virion release from the cell (Klimkait *et al.*, 1990). Assembly of the virions occurs at the cell membrane where the RNA, RT and core proteins associate with viral envelope glycoproteins and the immature viruses bud from the cell membrane (Schnittman & Fauci, 1994). Maturation of the virus particles takes place after budding. HIV proteases cleave the polyproteins into functional proteins and the structural components form the mature virion; gag is cleaved to form the p17 matrix, the p24 capsid, the p9 nucleocapsid and the p6 proteins and organisation of the viral core is completed (Gottlinger *et al.*, 1989).

1.5.4 Primary and Secondary Receptors

As described above, viral attachment to a susceptible cell is initiated by the binding, with high affinity, of the HIV envelope glycoprotein gp120 to the CD4 molecule (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984) which is expressed on the surface of many cell types including T lymphocytes, dendrocytes and monocyte / macrophages. The CD4 molecule is a glycopolypeptide with four immunoglobulin-like domains, the outer V1 immunoglobulin-like domain of which binds the gp120 molecule (Capon and Ward, 1991).

Some CD4-expressing human and animal cells are resistant to infection by HIV-1 and syncytial formation (Maddon *et al.*, 1986; Ashorn *et al.*, 1990; Chesebro *et al.*, 1990). Their research has shown that the CD4 molecule was essential for entry of HIV-1 into lymphocytes and that other cells which expressed CD4, either naturally or after transfection with the CD4 gene, bound the virus but did not become infected. These studies, and further experiments with animal cells transfected with the CD4 gene and fused with human cell heterokaryons, re-enforced the idea that the non-human CD4+ cells lacked human-specific cofactor(s) required for fusion with HIV-1 expressing cells (Maddon *et al.*, 1986; Ashorn *et al.*, 1990; Chesebro *et al.*, 1990; Dragic *et al.*, 1992; Broder *et al.*, 1993).

Feng *et al.* reported that laboratory adapted viruses were able to infect a variety of non-human cells which co-expressed CD4 and a G protein-coupled seven-transmembrane receptor, named HUMSTSR or LESTR, of unknown function (Feng *et al.*, 1996). They also showed that CD4 cells which expressed this protein, which they termed "fusin", could be fused with HeLa cells expressing the envelope of HIV-1. Feng *et al.* drew attention to the sequence similarity between fusin and the receptor for interleukin 8, an α-chemokine (CXC). Antibodies to fusin (CXCR4) inhibited infection and cell fusion by T-tropic viruses but not by macrophage-tropic viruses (Feng *et al.*, 1996; Hori *et al.*, 1998). In contrast, infection by macrophage-tropic primary viruses but not T-tropic viruses can be inhibited by β-chemokines RANTES, MIP-1α and MIP-1β (Cocchi *et al.*, 1996). Paxton *et al.* (1996) then suggested that some persons who remained sero-negative even after multiple exposures to HIV could be due to the expression of high levels of these chemokines by their T cells. They showed that the β-chemokines inhibited the *in vitro* infection of T cells by primary viruses but not laboratory adapted viruses. The findings of Paxton *et al.* (1996) indicate that, in addition to the CD4 glycoprotein receptor, macrophage-tropic viruses utilize a second cell surface molecule which is distinct from CXCR4. Alkhatib *et al.* (1996) and Dragic *et al.* (1996) also showed that infection of CD4 cells by macrophage-tropic, but not T-tropic viruses, was inhibited by β-chemokines, high levels of which are secreted by CD4 and CD8 T cells. They and several other groups reported that transfection of β-chemokine receptor genes into CD4-expressing cell lines made them susceptible to infection by macrophage-tropic variants. Choe *et al.* (1996) compared the efficiency of infection of recombinant HIV-1 viruses in HeLa-CD4 cells transfected with β-chemokine receptors 1 to 5 (CCR1 - CCR5). CCR5 was more effective than CCR3, supporting infection by a wider range of variants, including those in clades A, B, E and F: the other receptors were inactive. As noted above they showed that the structure of the V3 loop was an important determinant in the interaction between the virus and the chemokine receptors. Similar results were obtained by Doranz *et al.* (1996) who showed that CD4 target cells transfected with CCR5, but not CXCR4, were fused

by cells expressing glycoproteins from macrophage-tropic viruses. The laboratory adapted HIV-1_{IIIB} glycoprotein only responded to CXCR4 and a dual-tropic primary strain which replicates in both macrophages and T cell lines utilized both CXCR4 and CCR5: the V3 loop sequence of this strain was intermediate between those of M- and T-tropic viruses (Doranz *et al.*, 1996).

Samson *et al.* (1996) and Dean *et al.* (1996) reported that two persons who remained seronegative after repeated exposure to HIV were homozygous for a deletion in their CCR5 gene, which resulted in the production of a non-functional receptor. Their evidence indicated that persons who were heterozygous for this deletion also had some protection against infection. This deletion appears to be confined to Caucasians and would not confer protection to Africans, including the repeatedly exposed but seronegative members of the MRCPA cohorts in Uganda.

The hypothesis that primary, macrophage-tropic, NSI viruses utilize CCR5 and related β-chemokine receptors and that SI variants and cell line adapted viruses utilize α-chemokine receptors now appears to be an over-simplification as the observed receptor usage of some NSI variants does not correlate qualitatively with their cell tropisms *in vitro* (Cheng-Mayer *et al.*, 1997). For example, some virus isolates can use both CCR5 and CXCR4 but are unable to infect macrophages. Simmons *et al.* (1996) found that several dual-tropic SI primary isolates, which grew equally well in T cells and macrophages, could utilize either CXCR4 or CCR5 to infect CD4 cells. Further, 3 macrophage-tropic SI strains utilized CXCR4 but not CCR5. They and others have suggested that other co-factors or receptors may be used by some strains of HIV-1. Schmidtmayerova *et al.* (1996) reported that although β-chemokines inhibit the infection of T cells by primary viruses, they stimulated their growth in macrophages: conflicting data, which depended on culture conditions, was obtained for the inhibition of HIV replication in dendrocytes with β-chemokines (Rubbert *et al.*, 1997).

There is growing evidence, summarized by Endres *et al.* (1996) that some HIV isolates can infect a variety of cells in the absence of CD4: generally, HIV-infection of CD4- cells proceeds slowly and there are no cytopathic effects. However, Endres *et al.* (1996) showed that some strains of HIV-2 can use CXCR4 to infect CD4- human and animal cells productively and that the CD4-independent infection was inhibited by a monoclonal antibody specific for CXCR4. In an interesting parallel with the effect of HIV-1 infection on the expression of CD4, the expression of CXCR4 at the cell surface was down-regulated by infection with HIV-2. However, there is some evidence that the ability of HIV-2 to replicate in CD4- cells depends on the presence of mutations in envelope and transmembrane glycoproteins (Reeves and Schulz, 1997).

Clearly, research on the roles of different receptor molecules and other co-factors in HIV infection is still at an early stage. Dittmar *et al.* (1997) have shown that immune escape from neutralizing antibodies may result in changes in receptor usage. The fairly simple model of disease progression involving mutations within the viral glycoproteins, especially the V3 region, leading to different receptor/co-factor usages associated with changes in cell tropisms and widening of the range of cells and tissues susceptible to infection needs to be proven, especially with viruses which have not been selected by culture *in vitro*.

1.6 Molecular Biology

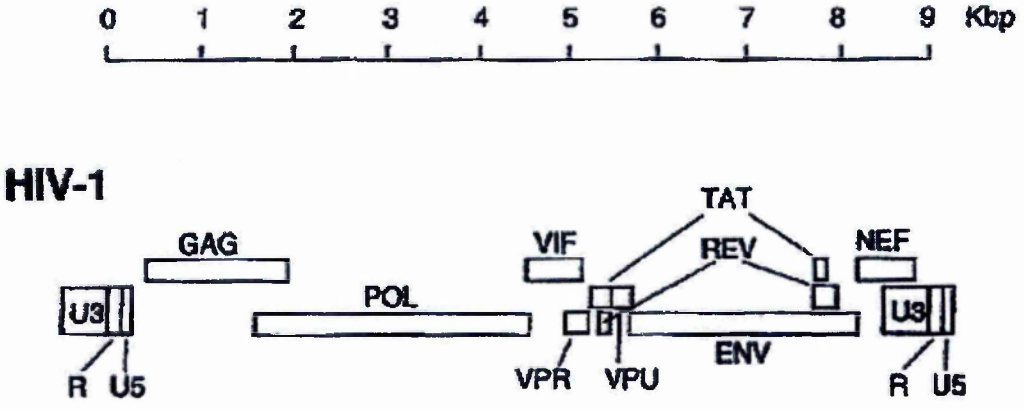
1.6.1 The Genome

Like other retroviruses, the lentivirus genome consists of two copies of RNA. These RNA encode the structural proteins which form the viral core and the surface envelope glycoprotein plus several enzymes, and regulatory or accessory proteins as described above.

The molecular organization of the genome of HIV-1 is shown in figure 5 (Myers *et al.*, 1993). The HIV-1 genome contains approximately 9.2 kilobases of RNA (or of DNA in the integrated provirus); the size varies between isolates, depending on the presence of insertions or deletions. A block of approximately 680 bases at

Figure 5. The molecular organization of the genome of HIV-1. The locations of the viral structural and accessory genes and the long terminal repeats (U3RU5) are indicated (Myers *et al.*, 1993).

Figure 5


0
1
2
3
4
5
6
7
8
9
Kbp
HIV-1
GAG
POL
VIF
TAT
REV
NEF
U3
R
U5
VPR
VPU
ENV
U3
R
U5

the 5' end are directly repeated at the 3' end of the genome to form the Long Terminal Repeat (LTR).

The *gag* and *env* genes encode the core and envelope proteins, respectively and the *pol, vif, vpu, vpr, tat, rev,* and *nef* genes encode reverse transcriptase, integrase and other non-structural or accessory proteins. The LTR functions as a powerful viral transcriptional promoter, the activity of which is greatly enhanced by factors produced during cell activation.

There are numerous splice donor and acceptor sites which may be used to give a series of RNA transcripts, as shown in figure 6 (Myers *et al.*, 1993). The *gag* and *pol* genes are not spliced and each is transcribed to produce polycistronic mRNAs which encode the core and polymerase proteins, respectively. Both proteins are cleaved by the virion protease, encoded by the pol gene, to produce several proteins: the *gag* gene product is cleaved into the p17, p24, p7 and p6 structural proteins and the *pol* gene product is cleaved to give the viral reverse transcriptase (RT) protease and integrase enzymes. The *env, vif, vpr, tat, vpu, rev,* and *nef* gene products are produced from spliced transcripts encoded by the 3' part of the genome. The *env* gene encodes the precursor glycoprotein gp160 which is cleaved by host cell enzymes into glycoproteins gp120 and gp41 (see above).

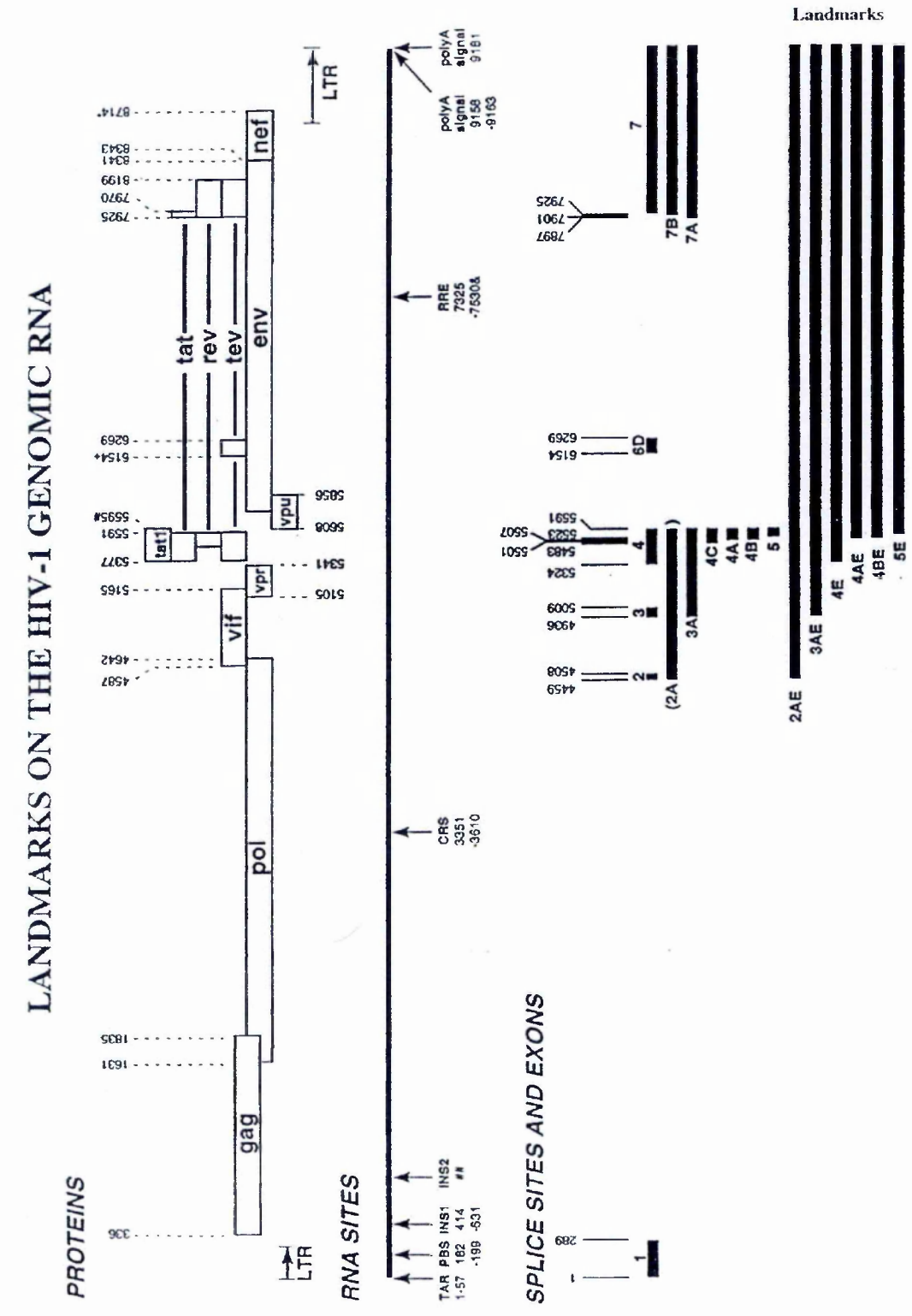
1.6.2 Genetic Diversity

The process of DNA synthesis by reverse transcription is highly prone to error, and in the absence of a proof-reading mechanism for correcting the mistakes, the mutation rate of HIV-1 is very high (approximately 1 mutation per replication cycle) (Ewald, 1994).

Mutations can be point mutation, insertions, deletions or due to recombination events. Point mutations, where one nucleotide is substituted for another, may result in the alteration of the amino acid sequence of the protein. Substitutions may cause the insertion of stop or nonsense codons in the genetic sequence,

Figure 6. Functional organization of the HIV-1 genome. The figure shows the splice donor and acceptor sites used to give a series of RNA transcripts which are translated into viral proteins (Myers *et al.*, 1993).

Figure 6


LANDMARKS ON THE HIV-1 GENOMIC RNA
PROTEINS
RNA SITES
SPLICE SITES AND EXONS
Landmarks
LTR
gag
pol
vif
vpr
tat
rev
tev
env
vpu
nef
LTR
TAR PBS INS1 INS2
1-57 182 414 #
-199 -531
CRS
3351
-3610
RRE
7325
-7530&
polyA signal 9158 -9163
polyA signal 9181
2AE
3AE
4E
4AE
4BE
5E
7B
7A
6D
4C
4A
4B
5
3A
(2A
2
3
4
7
1
-

which can block protein synthesis. Insertions and deletions of one or more nucleotide may also occur, these can cause reading-frame shifts and may also block protein synthesis. Recombinations can take place between strands of viral DNA and during reverse transcription. In persons where multiple infection has taken place template switches may result in the sudden exchange of large amounts of the genetic material between two isolates, which may be from distinct HIV-1 subtypes (Birx *et al.*, 1996). High virus turnover (Ho *et al.*, 1995) leads to an accumulation of errors in the viral genome and rapidly results in the formation of a "swarm" of variants, some of which may escape immune detection (Coffin, 1992; Bruce *et al.*, 1993). The rate of variation is not equal through out the genome; the structural genes *gag*, *pol* and *env* have differing rates of substitution with *env* being the most variable. Within the *env* gene there are five hypervariable regions (V1 - V5) and five constant regions (C1 - C5). It has been shown that amino acid sequence variations caused by mutations in the V3 loop coding region of the *env* gene can lead to alterations in the antigenicity, tropism and phenotype of the isolate. It seems likely that the same genetic variation which allows a variant to escape immune detection may also alter its cell tropism and lead to wider distribution of the virus in the body (Bonhoeffer *et al.*, 1995).

Based on *gag* and *env* coding sequences, 3 groups and a number of clades or subtypes of HIV-1 have been identified. The major group of HIV-1 isolates, or group M, contains 9 clades that have been labelled A, B, C, D, F, G, H, J, and K (McCutchan *et al.*, 1996; Triques *et al.*, 2000). Isolates originally grouped into clades E and I have been reassigned as circulating recombinant forms (CRFs) with mosaic genomes of more than one subtype. The clade E isolates have all been renamed as CRF01_AE (Robertson *et al.*, 1999) and the clade I isolates are now known as CRF04_cpx viruses (Nasioulas *et al.*, 1999). Each clade contains a number of viral isolates that are genetically related to each other and the clades are distinguishable from each other by nucleotide distances (Myers *et al.*, 1993; Birx *et al.*, 1996). In 1990 two HIV-1 isolates from the Cameroon were found to be genetically different from other HIV-1 isolates (De Leys *et al.*, 1991).

Further divergent isolates have been discovered which grouped with these viruses from the Cameroon, because of their overall genomic sequence differences from group M isolates they were labelled as outliers in the phylogenetic grouping of HIV-1 and became HIV-1 group O (Charneau *et al.*, 1994; Gurtler *et al.*, 1994). Phylogenetic analysis of an isolate of HIV-1 (YBF30) obtained in the Cameroon in 1995 revealed that it grouped between SIVcpz-gab and HIV-1 group M. This isolate and one other grouped together and were labelled the new non-M, non-O group of HIV-1: group N (Simon *et al.*, 1998).

The genetic diversity of HIV-1 is greatest in Africa and groups M, O and N and all of the clades of HIV-1 can be found in Africa (Loussert-Ajaka *et al.*, 1995; Louwagie *et al.*, 1995; Simon *et al.*, 1998; Vidal *et al.*, 2000). The global distribution of HIV-1 groups M, O and N and group M clades are shown in Figure 7 (Information for this figure was obtained via the www.hiv.lanl.gov website). Uganda is a country which shows a wide genetic diversity of HIV-1, where variants of clades A, B, C, D and G have been found, although the vast majority of variants are in clades A and D (Albert *et al.*, 1992; Bruce *et al.*, 1994; Kaleebu *et al.*, 1995). Analysis of the *env* genetic sequence of central and east African isolates has revealed many variations not seen in American and European clade B viruses.

1.7 Immunology

Many of the studies on the immunological responses to HIV infection have been influenced by the need to produce an effective vaccine or improve viral diagnosis. Exposure to HIV results in a three tier immune response; initially involving the non-specific activation of dendritic cells, macrophages, the release of cytokines and complement and the action of natural killer cells, followed by cytotoxic T cells and then neutralizing and other antibodies (Roitt, 1991; Oldstone, 1997; Palucka and Banchereau, 1999). In response to infection with HIV-1 the immune system produces antibodies to specific epitopes of the virus which direct the antibody-dependent cytotoxic cells to the

Figure 7. Global distribution of HIV-1 groups O, N and M and group M clades.

(Information for this figure was obtained via the www.hiv.lanl.gov website.)

Figure 7


B AG
B
B F
A B F H AE
B
A B F AE
B F
A B C
B F G
A B C D AB
B
B C F G AE
B AG
B G
C D G
G
A B G H AB
B
B G AE
A B C
B AE
A B F G K AG AE O N
A C D
G C D
B C AE
B
B C

virus infected cells (Broliden *et al.*, 1996). Cell mediated immune (CMI) responses are generally thought to be responsible for the control of intracellular infections and the clearance of infected cells. The cellular response to an acute viral infection may clear the target organ of virus before the neutralizing antibody titres are raised (Nye and Parkin, 1994). Infection with HIV-1 results in specific CTL responses and virus-induced antibody production. Although little is known about the mechanisms of protection against viral infection or diseases it is possible that protection may depend on the combined activities of cell mediated and humoral immune responses. The presence of neutralizing antibodies is generally regarded to be an important component in the immune system and it has been assumed that a vaccine able to induce a broadly neutralizing repertoire of antibodies would give some protection against HIV-1 infection. Therefore it is important to measure the neutralizing activities of antibodies induced both by vaccination and by natural infection with HIV and, where possible, to relate these activities with those of other immune systems, e.g. cell mediated immunity and the extent of protection or rate of disease progression.

The need to produce sensitive, specific and reliable serological methods for the diagnosis of HIV has lead to intense research and development activity, much within commercial and public health sectors, which fall outside this review. However, much work has been done to develop serological methods which could be used as research tools, for example in clinical epidemiology of HIV in developing countries where a simple assay to determine virus subtype would be invaluable. This was one of the major objectives of the work described in this thesis.

1.7.1 Serology and Sero-surveillance

Sera from HIV-infected persons react with most or all of the virus-induced glycoproteins and proteins, including non-structural proteins. Some of the glycoprotein epitopes, for example those in the V3 loop region of the gp120 and in the transmembrane glycoprotein, gp41, induce a very strong serological

response in almost all infected people and are especially useful in diagnostic studies.

Solid phase enzyme-linked immunosorbent assay (EIA) methods using synthetic peptides, chiefly from the V3 loop region, have been developed to identify the epitopes recognized by antisera. This system has been used to survey antisera from different geographical locations to see if the serological responses reflected the sequence or subtype differences and to determine whether simple serological assays could be developed into a sero-epidemiological tool (Blomberg *et al.*, 1993). As described in this thesis, work carried out using a simple EIA with V3 loop peptides led to a confusing array of cross-reactivity as the African antisera reacted with more than one synthetic peptide, often from subtypes other than that of the infecting isolate. However, antisera from asymptomatic persons infected with HIV-1 gave less cross-reactivity, indicating that this system could be usefully developed (Smith *et al.*, 1994). A study carried out with antisera from Uganda, Rwanda, Thailand and Brazil identified cross-reactivities between genotypes A and C and between genotypes B and D; Ugandan genotype D caused the most cross-reactivity (Cheingsong-Popov *et al.*, 1994). Antisera from the genotypes A and B in Thailand were serotyped using 14mer peptides representing the amino acids spanning the V3 loop apex. In this study highly concordant results were obtained between serotype by the V3 loop EIA and genotype by PCR (Pau *et al.*, 1993). In work carried out with Ugandan sera has suggested that the V3 loop of the gp120 is the major antigen of isolates from clades A, C and D (Pestano *et al.*, 1995). In a study by Kaleebu plasma from Ugandans infected with clade A, C and D isolates were serotyped by V3 EIA using 14 to 16 amino acid peptides spanning the loop apex: Although clades A and D could be distinguished from each other, clades A and C were indistinguishable by this method (Kaleebu, 1995). Introduction of a peptide blocking stage in which the sera are pre-incubated with excess peptide in solution has been shown to give a more specific EIA which may be used at different disease stages (Barin *et al.*, 1996).

The nature of the antibody response to the V3 loop over the time course of HIV infection was examined by a group from the Netherlands (Zwart *et al.*, 1992). Antibodies produced at the time of seroconversion were found to react in an EIA specifically with the V3 loop peptide with an amino acid sequence corresponding to that of the infecting virus isolate. The investigation of follow-up samples revealed that the levels of peptide reactivity increased and more cross-reactivity was seen during the 5 years after seroconversion. However, despite this broadening of the antibody response the peptide specificity (i.e. the peptide with which the antiserum reacted most strongly at seroconversion) remained unchanged. Only very rarely was there a change in antiserum specificity. At the later stages of infection antiserum reactivities with V3 loop peptides did not reflect the V3 loop sequences of the virus isolates circulating at that time. At the later stages of infection antiserum reactivities were strongest with the V3 loop peptides representative of the isolate present at seroconversion (Zwart *et al.*, 1992).

1.7.2 Neutralizing Antibodies

The immune system produces neutralizing antibodies which bind to cell-free virus preventing entry of virus into target cells and fusion of virus infected cells with uninfected cells. The principal neutralizing determinant has been identified as the V3 loop of the envelope glycoprotein in subtype B isolates of HIV-1 (Palker *et al.*, 1988; Rusche *et al.*, 1988). Peptides which mimicked the amino acid sequence of the V3 loop of a virus isolate were able to absorb the neutralizing antibodies from the antisera (Javaherian *et al.*, 1989). The addition of homologous V3 loop peptide to a neutralization system reduced the antiserum neutralizing effect on isolates HIV-1_{MN} and HIV-1_{IIIB} (Carrow *et al.*, 1991): These experiments confirmed the identification of the PND in subtype B isolates. Inoculation of laboratory animals with V3 loop peptides has been shown to produce antibodies capable of neutralizing HIV-1 isolates *in vitro* (Kenealy *et al.*, 1989; Laman *et al.*, 1992).

It has been shown (Oram *et al.*, 1991; Albert *et al.*, 1992; Bruce *et al.*, 1993, 1994; Myers *et al.*, 1993) that many African isolates of HIV-1 encode V3 loop sequences, which are dissimilar from those of North American and European viruses. Although it has not been shown that the V3 loop epitopes of African variants of HIV-1 induce neutralizing antibodies or cell mediated immunity, it is probable that this V3 loop region encodes important immunogenic epitopes in all variants of HIV-1.

Early virus neutralization experiments were done using laboratory adapted viruses grown on various T cell lines and sera from infected persons. Later, peptides encoded by *env* gene sequences of subtype B laboratory adapted viruses were used to produce antibodies in laboratory animals. Antibodies raised by injecting goats with V3 loop peptides encoded by HIV-1_{IIIB} and HIV-1_{RF} had isolate-specific neutralizing and SI inhibiting activities (Palker *et al.*, 1988). Neutralization of two subtype B virus isolates (HIV-1_{MN} and HIV-1_{IIIB}) by a six amino acid peptide (GPGRAF) which formed the apex of the V3 loop was seen in another study, but the apex regions of both isolates were the same (Javaherian *et al.*, 1990). Many other groups also recorded type-specific neutralization using cell line adapted viruses (Goudsmit *et al.*, 1988; Haigwood *et al.*, 1990; Arendrup *et al.*, 1993; White-Scharf *et al.*, 1993).

To determine whether a V3 subunit vaccine could induce group specific neutralizing antibodies, Wagner *et al.* synthesised a 36mer V3 consensus peptide based on the sequences of HIV-1 isolates from America, Europe and Africa (Wagner *et al.*, 1992). This peptide was used to demonstrate good cross-reactivity with a range of antisera of different subtypes and also to neutralize HIV-1_{IIIB} on the CEM cell line. It was also shown to induce specific cytotoxic T lymphocytes in mice.

Low passaged primary isolates are thought to resemble most closely the viruses present in infected humans. Neutralization epitopes are less accessible on the envelope glycoprotein of primary isolates of HIV-1 than on the cell line adapted strains (Moore and Ho, 1995). Many studies have shown correlation between antibody affinity for the mature processed oligomer of gp160 and neutralization

of virus (Fouts *et al.*, 1997; Roben *et al.*, 1994). The cell line adapted viruses and low passage primary isolates grown on PBMC showed marked differences in their susceptibility to neutralization. Only about 30% of primary isolates could be adapted to grow on T cell lines, and primary isolates have been shown to be less sensitive to neutralization with soluble CD4 (Daar *et al.*, 1990; Ashkenazi *et al.*, 1991; Moore *et al.*, 1992). In light of these discoveries it was thought that antibody neutralizations should also be carried out using primary isolates to reduce the possibility of misleading results (Matthews, 1994; Sawyer *et al.*, 1994). In an attempt to address the issue of possible differences between the cell line adapted and primary virus isolates Mascola *et al.* (1994) carried out a series of experiments using Thai and North American primary isolates from subtypes A and B grown on PBMC. These isolates were also adapted for growth on cell lines to allow definitive comparisons of the two systems. Overall type-specific neutralization was seen, and the primary isolates were less susceptible to neutralization. Work done with two Ugandan primary isolates on PBMC also resulted in type-specific neutralization (Atkin *et al.*, 1993).

Cross-clade neutralization of primary isolates on PBMC has been reported by a few groups (Trkola *et al.*, 1995; Mascola *et al.*, 1996a; Moore *et al.*, 1996). There has been an extensive neutralization study involving primary isolates from all of the 9 subtypes of the major group of HIV-1 using PBMC where an attempt was made to establish a correlation between genotype and "neutralization serotype" (i.e. the susceptibility of an isolate to serum neutralization). Five neutralization serotypes were identified, and these did not correlate with the genotypes of the isolates with the exception of the subtype E isolates from Thailand in which a correlation was found (Kostrikis *et al.*, 1996). The cross-clade neutralization and identification of neutralization serotypes may be an important step in the approach to developing a vaccine able to neutralize a large percentage of HIV-1 isolates world wide.

Much of the antibody response to the envelope glycoprotein may be raised not to mature oligomer, but to envelope glycoprotein that has become detached from the virion and which have increased exposure of antigenic epitopes

(Parren *et al.*, 1997). Immune response to these exposed epitopes on the monomeric or unprocessed glycoprotein may result in a population of memory B cells which is restimulated by exposure to less antigenic cross-reactive epitopes on mature glycoprotein, and the response to the less antigenic epitopes may be suppressed. This is known as original antigenic sin (Fazekas de St. Groth, 1966). In order to overcome this phenomenon and produce neutralizing antibodies a vaccine must present epitopes exposed on mature virions.

Near the C-terminal part of the extracellular gp41 there is a neutralization epitope, incorporating the linear sequence ELDKWA, that is conserved in many isolates of HIV-1. The human antibody 2F5 which maps to this linear sequence is broadly neutralizing *in vitro* (Muster *et al.*, 1994).

The mature oligomeric gp120 of cell line adapted isolates has two major exposed neutralization epitopes: the V3 loop and the CD4 binding site. Antibodies to these two epitopes neutralize cell line adapted isolates efficiently, though antibodies to V3 loop epitopes may be strain specific (Sattentau and Moore, 1995). The V3 loop of primary isolates is less accessible and therefore antibodies to this epitope are much less effective at neutralizing primary isolates (Bou-Habib *et al.*, 1994; D'Souza *et al.*, 1997). The CD4 binding site is also less accessible on the mature glycoprotein of primary isolates and antibody affinity to this epitope is much reduced in comparison to that on cell line adapted isolates (Sattentau and Moore, 1995). However two epitopes located near to these two sites with reduced accessibility in primary isolates have been identified by antibodies with strong neutralizing potential. One epitope involves residues at the base of the V3 loop in the C2 and C3 regions and the V4 region. The monoclonal 2G12, which has been mapped to this epitope, broadly neutralizes primary isolates (D'Souza *et al.*, 1997). The second epitope is located in part of the CD4 binding domain and is influenced by the V2 loop: monoclonal antibody b12 maps to this epitope and has been shown to broadly neutralize primary isolates cross-clade and from a range of geographical locations (Kessler *et al.*, 1997). However these two epitopes are not thought to be very

immunogenic, and antibodies b12 and 2G12 were obtained initially from a single infected donor.

Despite the low immunogenicity of HIV-1 envelope and limited accessibility of neutralizing epitopes, antibodies to the V3 loop and CD4 binding site have been used in passive immunization of chimpanzees and severe combined immunodeficiency mice with human peripheral blood lymphocytes; complete protection from cell line adapted isolates was achieved (Parren *et al.*, 1995). Such evidence suggests that antibodies are important in protection from HIV-1 infection.

Several groups have reported that some antibodies have an enhancing effect on the infectivity of HIV-1 and that these may play a role in the pathogenesis of HIV *in vivo*. In most cases enhancement by antibody involves complement (Robinson *et al.*, 1988a) whereas in animals infected with HIV-1 or SIV enhancement involves cells bearing F_c receptors (Takeda *et al.*, 1988; Homsy *et al.*, 1989). It was shown (Robinson *et al.*, 1988b) that antibodies in human sera could be separated into neutralizing and enhancing fractions by affinity chromatography, suggesting that the antibodies were induced by different antigenic domains. Two separate complement-dependent enhancing domains have been identified in the transmembrane gp41 (Robinson *et al.*, 1990) and antisera produced by vaccinating rabbits with peptides from many regions of the gp160, including the V3 loop, possessed enhancing activity for cell line adapted viruses (Jiang *et al.*, 1991). The role of enhancing antibodies in pathogenesis or immunity is unclear but they should obviously be taken into account in vaccine development studies.

1.7.3 Cell Mediated Immunity

There is evidence to suggest that major histocompatibility complex (MHC)-restricted HIV-specific CTL may have an important role in the immunological mechanisms of protection from infection by HIV and disease progression. Rowland-Jones *et al.* illustrated the presence of virus-specific CTL in an uninfected baby born to an infected mother (Rowland-Jones *et al.*, 1993).

A group of 10 nonprogressing HIV-1+ persons with stable CD4+ T cell counts of more than 500 cells/μl for 11 to 15 years were studied by Harrer *et al.* (1996). Eight of the ten nonprogressors had strong HIV-1 specific HLA class I-restricted CTL, recognising epitopes in gag, RT, nef and env proteins. A subset of these nonprogressors were found to have strong HIV-specific CTL activity, weak neutralizing antibody responses and a low viral load. Thus indicating that a cross-reactive neutralizing antibody repertoire is not necessary for long term nonprogression. However, it could be possible that some as yet undefined immune function has limited the viral load in these nonprogressors, and this reduced viral burden has maintained the CTL responses to HIV.

It has been shown that some individuals do not produce antibodies following exposure to HIV, but do have a CMI response. A group of exposed, but HIV-uninfected women were studied in the Gambia three out of six of these women were found to elicit HIV-specific CTL in response to peptide presentation by the most common Gambian human leukocyte antigen (HLA) class I molecule, HLA-B35 (Rowland-Jones *et al.*, 1995).

Epitopes that are recognised by HLA class I-restricted HIV-specific CTL have been mapped to the V3 loop (amino acids 308-322) and to a region in the C-terminal part of gp120 (aa 381-392). Five gag-specific epitopes have been mapped; three to p24 and two to p14. Five RT-specific epitopes have been mapped to areas which were translated from conserved regions of *pol* (Walker and Plata, 1990).

Vaccination of macaques has been shown to stimulate nef-specific CTL, which caused suppression of SIV replication (Gallimore *et al.*, 1995). DNA vaccination of BALB/c, AKR or C57BL mice with recombinant vaccinia virus expressing HIV-2_{ROD} *env* resulted in cross-reactive CTL to HIV-2_{ROD} and SIV_{MAC} (Agadjanyan *et al.*, 1997). CTL specific to gag and nef proteins were detected in macaques mucosally vaccinated with a *nef* depleted SIV_{MAC} and protected from challenge with SIV_{MAC} (Cranage *et al.*, 1997).

The MRC funded a project to evaluate CTL responses in infected Ugandans exposed to HIV-1 clade A, B, C or D viruses: McAdam *et al.* (1998) found cross-

reactive CTL to gag p55, but no cross-reactivity with HIV-2. The cross-clade reactivity may be important in recombinant vaccine design, as it suggests that a vaccine may also induce cross-reactive CTL (McAdam *et al.*, 1998).

It was suggested that CTL might have had a role in the pathogenesis of AIDS. CD8+ CTL were thought to contribute to the depletion of CD4+ T cells by lysis of HIV infected cells and cells with gp120 bound to their surface (Walker & Plata, 1990). The role of CD8+ CTL in infected CD4+ T cell lysis was confirmed when Yang *et al.* (1996) studied the recognition and lysis of CD4+ T cells infected with HIV-1_{IIIB} by CD8+ HLA class I restricted, HIV-1 specific CTL clones. They showed that HLA A2- and B14- restricted CTL clones recognised the HIV-1 infected CD4+ T cells as the production of intracellular p24 increased and prior to the peak virus production from the infected cells. They demonstrated lysis of the infected CD4+ T cells by Gag, envelope and RT specific CTL clones, despite HLA class I down-regulation on the surface of the infected cells (Yang *et al.*, 1996).

Klenerman *et al.* (1996) proposed a model to investigate the effect of CTL lysis on CD4+ T cell depletion during HIV-1 infection. They found that the CD8+ CTL lysis of infected cells did not significantly increase the rate of CD4+ T cell depletion, but did reduce the amount of virus released from infected cells. Therefore they deduced that CTL-mediated lysis reduced viral load by limiting virus production from infected CD4+ T cells (Klenerman *et al.*, 1996).

Shankar *et al.* (1999) determined that the mechanism engaged by CD8+ CTLs for the lysis of HIV-infected CD4+ T cells was the granule exocytosis pathway. This involves the release of perforin and serine proteases into the intercellular space and results in target cell lysis (Kagi et al., 1994). The effects of CD8+ CTL lysis on uninfected bystander CD4+ T cells were examined and they concluded that minimal damage occurred to these cells (Shankar *et al.*, 1999). However Shankar *et al.* (1999) did find that HIV-infected CD4+ T cells did cause lysis of uninfected CD4+ bystander cells via the fas-mediated lytic pathway, which may contribute significantly to CD4+ T cell depletion *in vivo*.

The relative importance of humoral and cellular immune responses in host protection during initial infection with HIV and disease progression have yet to be determined.

1.8 Prevention and Cure

1.8.1 Chemotherapy

Antiviral chemotherapy is now widely used in developed countries, and as our improved understanding of HIV pathogenesis emerges it has become apparent that antiviral therapy should be administered as soon as the CD4 cell count reaches 350 cells per mm^3 in order to reduce the viral load and the distribution of HIV in the body (Volberding, 2003). It has been observed that a number of antiviral drugs lead to the rapid selection of resistant variants and also to intolerance by the patient. In response to these problems combination drug therapies are on trial and new antiviral agents are being developed (Eron, 1996; Hammer, 1996; Pennisi & Cohen, 1996; Trono, 1996; Kirk, 1998; Volberding, 2003).

Five classes of antiretroviral drugs are currently available for the treatment of HIV infection: Nucleoside reverse transcriptase inhibitors, nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors and fusion inhibitors (De Clercq, 2004a).

Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs and NtRTIs) are aimed at the reverse transcription stage of the virus replication cycle. These drugs are substrate analogues which are incorporated into the DNA by reverse transcriptase and terminate DNA elongation (De Clercq, 1992). Many of these analogues are in use or in clinical trials (e.g. zidovudine, didanosine, lamivudine, stavudine, lamivudine, abacavir, emtricitabine and NtRTI tenofovir disoproxil fumarate).

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind to reverse transcriptase close to the nucleoside binding site and slow down polymerization

(De Clercq, 1992). Three NNRTIs are available for therapy - nevirapine, delavirdine and efavirenz.

Protease inhibitors (PIs) inhibit the unique retroviral protease which is required for virus maturation. PIs currently in use are indinavir, ritonavir, saquinavir, nelfinavir, amprenavir, lopinavir (with ritonavir) and atazanavir (De Clercq, 2004a).

The fifth type of antiretroviral drugs are fusion inhibitors (FIs) such as the licensed T-20 (Enfuvirtide) which prevents HIV binding to the host cell by binding to gp41 during fusion. The use of T-20 is limited to patients with viruses that are resistant to the drugs or combinations of the drugs mentioned above (Moyle, 2003).

Treatment with three of these drugs in a combination therapy, such as nevirapine / zidovudine / didanosine has been shown to reduce the plasma viral load to undetectable levels for prolonged periods in 75% of antiretroviral-naive individuals with CD4 counts of 200-600 cells/μl. This is an example of the type of triple combination therapy used to overcome the problems of drug resistance by viral variants (Hammer, 1996). Typical treatment in Western Europe is a combination of two nucleoside analogues and one protease inhibitor (Volberding, 2003). Highly active anti-retroviral therapy (HAART) has become the normal approach to anti-HIV chemotherapy as more effective combinations of drugs have been licensed for use. Further improvements of the drug regimens are being developed, such as single tablets combining more than one drug and once-daily dosage which inevitably increase patient compliance.

Future antiviral chemotherapies are under investigation, one of which is based on the suppression of CD4+ T cell activation by the cytokine interleukin-16 (Baier & Kurth, 1997). Integrase catalyses the integration of viral DNA into host cell DNA, a unique and essential step in viral replication. The oligomeric enzyme acts in the cell cytoplasm to process the viral DNA and then in the cell nucleus where integration takes place. MAP30 and GAP31 proteins and curcumine and doxorubicin form complexes with viral integrase and prevent integration in infected cells (Thomas and Brady, 1997). Integrase inhibitor S-

1360, a diketo acid derivative, is now in clinical trials (De Clercq, 2004b). Transcription inhibitors aimed specifically at the transactivation of the viral LTR by the Tat protein are another group of drugs under investigation. N-aminoimidazole derivatives (S-1153 and AG1549) and pyridine oxide derivatives (JPL-32, JPL-88 and JPL-133) may attribute part of their anti-HIV activities to the inhibition of Tat (De Clercq, 2004b). The use of chemokine receptors as secondary receptors for HIV cell entry (as discussed in section 1.5.4) has led to the development of therapies using modified chemokines and small molecule antagonists to prevent HIV binding with these receptors on the cell surface. Many companies are now researching chemokine-receptor blocking therapeutics (Cohen, 1997).

The global prevalence of HIV is illustrated in appendix I (UNAIDS and WHO, 2003), which identifies many developing nations with high numbers of infected persons. Many developing countries have poor medical health systems due to weak economies and unstable political situations. In practice this may mean that reliable distribution of drugs throughout some countries may not be possible, incorrect prescription of drugs may occur due to inadequate facilities for diagnosis and improper use of drugs by poorly educated patients is common (Heymann *et al.*, 1993; Stevens *et al.*, 2004). It is important to realise that in a weak economic environment expensive antiviral treatments are not available to the infected public as a whole and priorities centre on improving the medical infrastructure and increasing education about the transmission of HIV (Kengeya-Kayondo *et al.*, 1997). Most of the developing countries rely on international organizations to donate drugs or to provide them at reduced cost for the treatment of many HIV-related secondary infections. However, few drugs are available for the treatment of HIV infection and this has prompted the World Health Organisation to initiate an international effort to increase the availability of antiretroviral drugs to 3 million infected persons in developing countries by 2005 (Elliott, 2002). Some treatment programs or trials are underway in Africa to prevent mother to child perinatal transmission of HIV (Perez *et al.*, 2004; Taha *et al.*, 2004). Evaluation studies have been carried out in

some African countries and assessments are planned in others to enable wider access to therapy for HIV (Katzenstein *et al.*, 2003; Nguyen *et al.*, 2003; Kober and Van Damme, 2004; Zulu *et al.*, 2004).

1.8.2 Vaccines

The effectiveness of newer combinations of anti-HIV drugs may improve the outlook for the control of HIV infections by chemotherapy, however lengthy treatment periods are required and the drugs are relatively expensive, i.e. about $10,000 per person each year. The cost would be much too high for persons in developing countries which have the highest incidence of HIV. Therefore, the development of an effective vaccine is still very important for the global control of HIV. The preparations for AIDS vaccine trials in developing countries must respect the ethics of the host nation and consider the public reaction to AIDS vaccination (Katongole-Mbidde, 1994). Communication with the people of vaccine host nations and their education into the vaccination programme should be an important preparation stage for vaccine trials.

In order to assess the efficacy of an AIDS vaccine the use of an animal model has been essential. HIV-1 has been shown to infect chimpanzees, gibbon ape, and rabbit, but development of disease does not parallel that of humans. The chimpanzee species is endangered, thus massively limiting their experimental usage (Gardner and Luciw, 1989). The use of a rodent as an animal model for AIDS has been precluded due to the inefficient replication of HIV in rodent cells caused by post-transcriptional blocks (Bieniasz and Cullen, 2000; van Maanen and Sutton, 2003). FIV in cats can provide a model for HIV disease and development of this system to study the efficacy and immune correlates of vaccines is underway (Burkhard and Dean, 2003). SIV_{MAC} (closely related to HIV-2) infection of rhesus macaque monkeys leads to disease and this has been the basis of the development of the animal model for AIDS (Geretti, 1999).

Experiments carried out by Joag *et al.* (1997) in pig-tailed macaques have established that they may be used as a model for intravaginal transmission of HIV. A single intravaginal inoculation of a chimeric virus $SHIV_{KU-1}$ resulted in infection that led to AIDS in the animals. Chimeric viruses or SHIVs consisting of SIV_{MAC} genome with HIV gene insertions (including *tat, rev, vpu* and *env*) have been shown to infect macaques and produce neutralizing antibody and CTL responses (Joag *et al.*, 1997; Ui *et al.*, 1999). The use of SHIV chimeras allows testing of potential human vaccines in the macaque model (Barouch *et al.*, 2000).

Early vaccine development was centred on the use of the HIV envelope protein to produce protective neutralizing antibodies, more recently there has been a shift in focus towards the production of a cell mediated immune response (specifically CTLs) from vaccination. However it is now widely accepted that a successful vaccine for AIDS will have to stimulate the whole immune system; innate, humoral and cellular responses (Letvin, 1998; McMichael *et al.*, 2002). The major types of vaccines developed for HIV include chemically inactivated whole virus, antigen subunits, live attenuated virus, plasmid DNA with viral gene inserts and live recombinant vectors expressing viral DNA.

Vaccines based on chemically inactivated whole virus have been used successfully to prevent viral infection such as influenza. Early studies showed that formalin-inactivated whole SIV vaccine protected macaques from challenge with the same strain of SIV (Murphey-Corb *et al.*, 1989, Desrosiers *et al.*, 1989). A number of research groups followed different protocols. The strains of SIV varied, examples used included $SIV_{MAC}251$ and $SIV_{SM}E660$. A variety of vaccination regimens were carried out and the macaques were challenged with various doses of live virus via intravenous and mucosal routes. Cranage *et al.* (1992) showed protection of 4/4 macaques from intrarectal (mucosal) challenge with SIV after a series of intramuscular vaccinations. Putkonen *et al.* (1992) went on to demonstrate the use of whole

inactivated SIV_{MAC} to protect 4/4 macaques from heterologous challenge with SIV_{SM}.

The success of these whole inactivated virus vaccine studies was short lived as research revealed that protection was due to anti-cell immune responses. The vaccine strains of SIV and the challenge stocks were all grown on human cell lines. Therefore the virus membrane presented antigenic human cell proteins at its surface against which the macaques' immunities were raised, and hence protection was afforded. Experiments to confirm this suspicion showed that macaques vaccinated with whole inactivated SIV grown on human cells were not protected from SIV challenge with virus grown on macaque cells (Stott, 1991; Cranage *et al.*, 1993; Putkonen *et al.*, 1993; Goldstein *et al.*, 1994).

Antigen subunit vaccines for AIDS have also been investigated. They consist of an immunogenic carrier in combination with a viral protein (mainly envelope glycoproteins). Studies in macaques with SIV subunit vaccines have been carried out following some early promising work by Berman *et al.* (1990) in which 2 chimpanzees were protected from homologous HIV-1 infection by vaccination with recombinant gp120. A glycoprotein enriched subunit vaccine protected 2 of 4 macaques from homologous SIV infection in research by Murphey-Corb *et al.* (1991). Giavedoni found that vaccination with SIV_{MAC} gp130 alone did not protect macaques from viral challenge. However, when SIV_{MAC} gp130 was used as a boost in conjunction with recombinant vaccinia virus expressing SIV_{MAC} gp130, then the viral load of the macaques after virus challenge was reduced (Giavedoni *et al.*, 1993). Similarly, reduced viral load and partial protection from infection was achieved after vaccination with gag and env proteins and with env proteins alone by Israel *et al.* (1994). In a more recent study 8 macaques were protected from homologous challenge with SHIV-W6.1D after vaccination with recombinant HIV-1 gp120 in SBAS2 adjuvant. All the protected animals had anti-gp120 antibodies and virus neutralizing antibodies after vaccination, but pre-challenge only 3 of the 8 had intermittent

CTL responses (Mooij *et al.,* 1998). Further development of the research into subunit vaccines is required to increase the vaccine antigenicity and to establish correlates of immunity. Towards this end the production of oligomeric envelope glycoproteins is being optimised. It is hoped that oligomeric conformations will give rise to antibodies capable of neutralizing primary virus isolates. If so, then they may play an important role in conjunction with DNA vectors in bimodal vaccines for HIV.

Live attenuated virus vaccines rely on *in vitro* laboratory propagation of replication competent virus with reduced pathogenicity and they have been used successfully to prevent virus infections such as measles, polio and chicken pox. Initially studies with SIV attenuated by deletions in the *nef* gene showed that macaque monkeys could be protected from subsequent wild-type virus infection (Daniel *et al.,* 1992). Use of the macaque model has led to the discovery that vaccination with live *nef* attenuated SIV_{MAC} will protect macaques from intravenous challenge with pathogenic SIV_{MAC}, by rectal mucosal challenge and from cross-strain challenge (Cranage, 1993; Cranage *et al.,* 1997). In a SIV vaccine study with a 12 base pair deletion in *nef,* repair of the deletion occurred by a sequence duplication event and subsequent sequence mutation. The gene repair coincided with a reversion to virulence and resulted in an increased viral load and consequent disease progression (Whatmore *et al.,* 1995). Studies in this line of vaccine development showed that the attenuated SIV vaccine strain caused AIDS and death in neonatal and adult macaques (Baba *et al.,* 1995; Hofmann-Lehmann *et al.,* 2003). In contrast, work carried out by Desrosiers (1998) with SIV attenuated by *nef* deletion and by *nef* and *vpr* deletions has not led to disease in macaques after 9 years of follow up. However there are still some safety concerns regarding the possible pathogenicity of such attenuated viral strains, and many unanswered questions about the long-term efficacy of vaccinating people with live attenuated strains of HIV. There is an ongoing study of just such a scenario in Australia of 7 people infected with a naturally occurring HIV-1 with deletions in *nef* and in the LTR part of the viral genome. These infected

persons had maintained low levels of plasma viraemia for between 13 and 17 years and had no disease progression (Dyer *et al.*, 1999). More recently the CD4+ lymphocyte counts of 3 of them have declined significantly perhaps signalling the start of immunodeficiency and disease progression (Learmont *et al.*, 1999). The safety concerns seen in the attenuated SIV vaccines would appear to be extended to the attenuated human HIV infections. However these attenuated virus studies have revealed a wealth of information about the immunopathogenicity of the AIDS virus and are of value in the elucidation of the correlates of protection (Whitney and Ruprecht, 2004).

Limitations with the vaccination approaches described above have led to research into new DNA vaccine constructs including the development of plasmids and live recombinant vectors to deliver viral immunogens more capable of raising humoral and cellular responses. The development of the "prime-boost" concept of vaccine delivery has increased the efficacy of these recombinant vaccines. Priming with a recombinant vector (such as a DNA plasmid with the viral gene) initially stimulates the CTL response to the expressed protein. These cellular responses are then expanded when the boost vaccination of a different recombinant vector construct (such as a replication-defective pox virus vector expressing the viral protein) occurs a few weeks later (Woodland, 2004).

DNA plasmids expressing HIV or SIV genes have been developed for use as AIDS vaccines or as a component thereof. The plasmids vary enormously, use different delivery systems and express different HIV or SIV genes. Early work by Wang *et al.* (1993) demonstrated the potential of DNA vaccination for AIDS. Wang *et al.* showed that 8 of 10 BALB/c mice inoculated with plasmid DNA expressing HIV-1_{HXB2} gp160 elicited cellular and humoral immune responses. Antisera from 8 of the mice neutralized HIV-1_{IIIB} in MT-2 cells and inhibited CD4-binding. Lymphocyte proliferation to recombinant gp120 (rgp120) was also observed. Lu *et al.* (1996) vaccinated 7 macaques with a plasmid expressing a

combination of 5 SIV_{MAC} genes by intramuscular, intravenous and gene gun routes. One plasmid encoded an 8.2kb $SIV_{MAC}239$ insert and expressed non-replicating virus particles. The other 4 plasmid constructs expressed SIV envelope glycoproteins. Throughout the vaccination regimen the immune responses of the macaques were monitored; transient neutralizing antibodies were elicited in all 7 macaques and persistent CTL responses to env were raised. However viral challenge with $SIV_{MAC}251$ led to infection in all monkeys and to CD4+ cell loss subsequently. In the vaccinated macaques as compared to the control group the acute viral stage was foreshortened, but chronic infection was not prevented and viral loads in this latter stage were not reduced. Egan *et al.* (2000) showed that 3 of 4 macaques had much reduced viral loads following virus challenge after SIV *gag* DNA plasmid vaccination. This study correlated gag-specific CTL response with protection from disease progression. Barouch *et al.* (2000) initially showed protection from disease after SHIV-89.6P challenge in all 8 macaques vaccinated with an $SIV_{MAC}239$ *gag* and HIV-1 89.6P *env* expressing DNA plasmid coupled with an IL-2-immunoglobulin cytokine fusion protein or with an IL-2-immunoglobulin plasmid. Seven of the 8 macaques have remained disease free for 2 years post-challenge. One macaque died after 52 weeks of AIDS after viral mutation in *gag* allowed escape from CTL recognition (Barouch *et al.* 2002). The death of this monkey exposes a worrying limitation of the protection offered by this type of vaccine. Perhaps more promising was the small pilot study carried out by Letvin *et al.* (1997) with only 2 rhesus macaque monkeys. The main aim of the study was to examine the humoral antibody titres elicited in response to plasmid DNA vaccines expressing HIV-1_{HXBc2} derived *env* (as gp120 and membrane bound gp160) and a boost of HIV-1_{IIIB} gp160. The gp120 binding antibody titres rose considerably after the protein booster. Pre-boost antisera did not neutralize $SHIV_{HXBc2}$ in MT-2 cells whereas post-boost antisera did. Env-specific CTL responses were also observed in both monkeys. Protection from disease following SHIV challenge was achieved over a 21 week follow up period. More importantly this small

study illustrated the potential of bimodal vaccination to extend the immune responses and to encompass both the cellular and the humoral elements.

A wide variety of live recombinant vectors expressing genes from AIDS viruses have been developed including alphaviruses, herpesviruses, recombinant yeast, Salmonella, Listeria, adenoviruses and poxviruses. After disseminated vaccinia infection in an HIV infected person (Redfield *et al.*, 1987) safety concerns about vaccinating immunocompromised persons with vaccinia virus has led to research into the efficacy of using attenuated poxviruses NYVAC, ALVAC and modified vaccinia Ankara (MVA) to deliver the immunogens. NYVAC originated from the vaccinia virus Copenhagen strain and has specific virulence and host range genes deleted from its genome rendering it safe to use in human vaccines (Paoletti, 1996). ALVAC is derived from canarypox virus and is unable to produce infectious virus progeny in mammalian cells (Baxby and Paoletti, 1992). MVA is attenuated vaccinia virus Ankara strain obtained after passage 500 times on primary chick embryo fibroblasts. MVA has multiple deletions in its genome and has very limited replication on human cells, enabling its use safely even in the immunocompromised host (Mayr *et al.*, 1978; Meyer *et al.*, 1991; Blanchard *et al.*, 1998). MVA was used at the end of the Smallpox eradication campaign to successfully and safely vaccinate more than 120000 people (Mayr *et al.*, 1978). In one study the use of MVA as a vector for SIV vaccine constructs led to persistent anti-vector antibody responses from all the macaques throughout the immunization regimen. These anti-MVA vector antibodies were associated with a reduction in the CTL response to the expressed SIV recombinant protein (Sharpe *et al.*, 2001). Adenovirus vectors such as those using adenovirus serotype 5 (Ad5) have also been studied for use as delivery systems for AIDS virus genes. However pre-existing immunity due to $CD8^+$ T lymphocytes and neutralizing antibodies to the Ad5 capsid in human populations limits the recombinant protein expression of the vector (Gahéry-Ségard *et al.*, 1998; Sumida *et al.*,

2004). Further development of novel adenovirus vectors with different capsid proteins may overcome this limitation (Gall *et al.*, 1998).

Much of the early vaccine research into the use of live recombinant vectors as vaccines for AIDS was carried out using small numbers of macaques and expression of SIV or SHIV genes. Israel *et al.* (1994) used 2 recombinant vaccinia vectors expressing $SIV_{MAC}BK28$ *gag* or *env* genes to prime 6 macaques and then boosted them with gag and/or env proteins. The 3 macaques which received the recombinant vector expressing SIV *env* and env protein had partial protection from disease following viral challenge; with reduced viral load in two and apparent clearance of virus from blood and lymph nodes in one. Of the 3 macaques which received the recombinant vector expressing SIV *gag* and *env* and the gag and env protein boosts only 1 macaque had a reduced viral load following viral challenge compared to the controls. All 6 macaques had neutralizing antibodies to env and those vaccinated with gag had neutralizing antibodies to gag. Lymphocyte proliferative responses were seen in 4 of the 6 macaques, but these did not correlate with protection from SIV challenge. Benson *et al.* (1998) inserted SIVK6W *gag, pol* and *env* into the NYVAC vector and administered this vaccine (NYVAC-SIV) to 24 macaques. Eight received NYVAC-SIV alone, 8 received NYVAC-SIV and NYVAC expressing interleukin-12 (NYVAC-IL-12) and 8 received NYVAC-SIV, NYVAC-IL-12 and NYVAC expressing interleukin-2 (NYVAC-IL-2). After 3 vaccinations the antibody titres of the macaques which had received NYVAC-SIV alone were 3 times higher than those of the macaques which had received the cytokine expressing vectors. The macaques which also received NYVAC-IL-12 had CTL responses after 3 vaccinations. Half of each group were challenged intravenously and half intrarectally (mucosally) with $SIV_{MAC}251$. All of the macaques that were challenged intravenously became infected. Five of 11 macaques challenged intrarectally were able to clear the virus and 6 of 11 became chronically infected, but their disease progression was slower than that of controls. In this study $CD8^+$ T-helper cell induction by vaccination

correlated with post-challenge viral load and slower progression to disease. Ourmanov *et al.* (2000) carried out a study involving 24 macaques with a lengthy follow up period. Three groups of 6 macaques were vaccinated with recombinant MVA expressing *gag, pol* and/or *env* genes from SIV_{SM}H-4 and 6 control macaques received non-recombinant MVA. Four weeks later they were challenged intravenously with the closely related SIV_{SM}E660. Following viral challenge all of the macaques became infected; the 18 which had received MVA-SIV recombinant vaccines had reduced plasma viral loads compared to the controls, and 4 of these became virus culture negative after 6 to 16 weeks. After 19 months post-challenge 10 of the vaccinated monkeys and 1 control monkey were still alive. Four of the surviving vaccinated monkeys had no apparent disease progression. Barouch *et al.* (2001) studied the immune responses and subsequent viraemia post-SHIV-challenge of 4 macaques immunized with recombinant MVA expressing SIV_{MAC}239 *gag-pol* and HIV-1 89.6 *env*. The vaccinated macaques elicited strong CTL responses and high titre neutralizing antibodies following the highly pathogenic SHIV-89.6 challenge and showed no signs of disease progression. In this study pre-challenge CTL responses correlated with reduced post-challenge viral loads. Amara *et al.* compared the immunogenicity and the protective efficacy of a combined DNA plasmid and a recombinant MVA vector vaccine with the recombinant MVA vaccine alone (Amara *et al.*, 2001, Amara *et al.*, 2002). One group of macaques was immunized twice with a DNA plasmid expressing SHIV-89.6 *gag, pol, vif, vpx* and *vpr* of SIV origin and *env, tat* and *rev* of HIV-1 origin. These were followed by a third immunization of the recombinant MVA booster expressing SHIV-89.6 *gag, pol* and *env*. A second group of macaques were immunized with of 3 vaccinations of rMVA. Seven months after the booster the macaques were challenged intrarectally with the pathogenic SHIV-89.6P. Comparison of the immune responses to the vaccinations by the 2 groups revealed that the rMVA only vaccine raised much lower levels of $CD8^+$ T lymphocytes and much higher titres of envelope binding antibodies than the DNA plasmid / rMVA vaccine combination.

After viral challenge all of the macaques became infected; the levels of infected CD4 cells was higher in the rMVA only vaccinated macaques and the cell free viral load was higher in the DNA plasmid / rMVA vaccinated macaques. Despite these differing patterns and kinetics of the immune responses to the two vaccine systems all of the vaccinated macaques controlled their infections to the limit of detection and maintained their CD4 cell counts.

Further understanding of the immune correlates of protection raised by experimental HIV vaccines is essential to the development of an effective prophylactic vaccine. In order to determine how far the safety, immunogenicity and protection seen in vaccine animal models is extended to the prevention of HIV infection in humans (or the slowing of disease progression) the next stage in vaccine development has been the commencement of human HIV vaccine trials. To this end the limitations of the animal models available are acknowledged and a number of candidate vaccines have been tested in human trials to establish their safety and immunogenicity.

A large number of candidate vaccines have been evaluated within the AIDS Vaccine Evaluation Group (AVEG) which was set up in 1990 to monitor Phase I clinical trials of 14 vaccines in HIV-uninfected recipients. The first 14 candidate vaccines contained the envelope glycoproteins, gp120 or gp160 singly or together, from cell line adapted viruses. The vaccines induced virus-neutralizing antibodies with strong neutralizing activities against homologous and, in some cases, heterologous cell line adapted viruses. However, none of the antibodies was able to neutralize primary HIV-1 isolates (Matthews, 1994; Wrin & Nunberg, 1994; Mascola *et al.*, 1996b). Whereas sera from naturally infected persons binds preferentially to native forms of gp120/160, sera from vaccinees preferentially bound to denatured forms of gp120, suggesting the lack of antibodies against conformational epitopes (VanCott *et al.*, 1995a). It seems probable that the vaccines had sub-optimal protein conformations that did not

mimic the oligomeric structure of gp120/gp41 in the envelope glycoprotein. Vaccination of rabbits with a highly purified oligomeric form of gp160 induced high titre antibodies, some of which neutralized some primary HIV-1 isolates (VanCott *et al.*, 1997). The inability of sera raised in vaccinees to neutralize primary isolates may be due to differences in the exposure of specific epitopes in these viruses, compared with the vaccine strain. This is evidenced by the inability of V3 loop-specific antibodies to recognise macrophage-tropic viruses (Bou-Habib *et al.*, 1994) and the lower neutralizing activity of V3 loop specific antibodies towards primary compared with the cell line adapted virus HIV-1_{MN} (VanCott *et al.*, 1995b). This parallels the differential susceptibilities of primary and cell line adapted viruses to neutralization by soluble CD4 (Daar *et al.*, 1990; Ashkenazi *et al.*, 1991; Moore *et al.*, 1992). As mentioned above, the possibility of misleading results should be minimised by using primary isolates instead of cell line adapted viruses in antibody neutralization assays, especially in vaccine studies (Matthews, 1994; Sawyer *et al.*, 1994).

Vaccine-induced CTL were found most frequently (15-40%) in recipients of the live vector vaccines, such as the recombinant pox vectors with HIV-1 *env* gene (McElrath *et al.*, 1997). The next stage of trials evaluated vaccines with DNA plasmid or live recombinant vectors expressing HIV *env* and other HIV genes. Recombinant Canarypox vaccine expressing gp160 or gp120 with *gag* / *pol* of HIV-1_{MN} were tested in humans; 90% developed antibodies and 40% CTLs (Oxford and Jeffs, 1996). The DNA plasmid vaccine trial carried out recently by MacGregor *et al.* (2002) had some unexpected results. Eighteen volunteers were vaccinated with a DNA plasmid expressing HIV-1_{MN} *env* and *rev* at 3 doses (100μg, 300μg and 1000μg). No antibodies were detected by ELISA to rgp120 of HIV-1_{MN} or rgp160 of HIV-1_{IIIB} following vaccination. CD4+ CTL responses to env were detected in the peripheral blood of 66% of the 12 vaccinees who received 300μg or 1000μg of the vaccine, and CD4+ CTL responses to rev were detected in the blood of 50% of these higher dosed groups. The complete lack of CD8+ CTL response in humans to this DNA plasmid was not anticipated.

However some encouraging results were obtained from a vaccine trial recently completed in Uganda, where an ALVAC vector expressing HIV-1_{MN} *env* (clade B), *gag* and *pol* (protease) was assessed for cross-clade immunogenicity to 2 of the predominant HIV-1 clades in the population (clades A and D). At present the recombinant genes are from clade B viruses and it is important that the vaccine-induced CTL should be able to recognise and lyse primary isolates of HIV-1 from various HIV-1 clades. Five of the 20 vaccinees in this trial had detectable CD8+ CTL responses following vaccination to clade B gag or env: All 5 had cross-clade reactivity with clades A and/or D. Three of the vaccinees had antibodies capable of neutralizing cell line adapted HIV-1_{MN} and the antibodies of 2 of these vaccinees were capable of neutralizing primary virus albeit at low titres. None of these antibodies were able to neutralize viruses from clades A or D (Cao *et al.* 2003). As demonstrated in this study, a single clade vaccine is able to raise cross-clade CD8+ CTL responses in a population where multiple viral clades are present.

In a recent vaccine study carried out in the United Kingdom a regimen of plasmid or MVA vector or a DNA-prime MVA-boost both expressing HIV-1 clade A Gag p24/p17 and a number of clade A CTL epitopes has been assessed. Twenty nine of the 35 volunteers elicited HIV-specific CTL responses to the vaccine (Mwau *et al.*, 2004).

In an earlier trial to investigate CTL responses 4 volunteers were vaccinated with ALVAC-HIV expressing $gp160_{MN}$ and boosted twice with rgp120 of HIV-1_{SF2} and 4 volunteers were vaccinated with ALVAC-HIV expressing $gp120_{MN}$ and gp41, *gag* and protease from HIV-1_{LAI} not combined with a booster of envelope glycoprotein. CD8+ CTL responses to envelope were detected in 100% of the vaccinees and in the group which received the multiple gene expressing vector 3 of 4 had CD8+ CTL responses to gag. The CD8+ CTL responses to NSI primary isolates from clades A to F were then assessed for 2 vaccinees from each group: one of samples from the ALVAC plus booster group showed broad cross-clade reactivity to all 6 primary isolates, the other did not recognise any. The samples tested from the ALVAC without booster group showed restricted

reactivity - one with isolates from clades A, B, C, D and E and the other with isolates from clades A, B, C and F (Farrari *et al.*, 1997). Clement-Mann *et al.* (1998) vaccinated 101 volunteers in 3 groups with either recombinant ALVAC expressing HIV-1_{MN} gp160 alone, ALVAC expressing HIV-1_{MN} gp160 with a booster of HIV-1_{SF2} rgp120 or HIV-1_{SF2} rgp120 alone. Neutralizing antibodies to HIV-1_{MN} and HIV-1_{SF2} were found in the sera from all of the ALVAC-gp160/rgp120 vaccinees, in less than 65% of ALVAC-gp160 alone vaccinees and in 89% of rgp120 only vaccinees. CD8+ CTL responses were detected in the peripheral blood of 37% of ALVAC-gp160/rgp120 vaccinees, 22% of ALVAC-gp160 alone vaccinees and in 10% of rgp120 only vaccinees. In another trial investigating this bimodal type of vaccine Evans *et al.* (1999) used ALVAC expressing gp120, gp41, *gag* and protease genes and CTL epitopes from *nef* and *pol* simultaneously vaccinated with or boosted with HIV-1_{SF2} rgp120 to vaccinate 119 volunteers. CD8+ CTL responses were elicited to a broad range of epitopes (env 22%, gag 32%, nef 16%, and pol 19%) and were detected for up to 10 months after the last vaccination. These trials indicate that immunogenicity may be significantly improved by optimisation of recombinant immunogens to include *gag* and other HIV genes with *env* and by their administration in combination with glycoprotein boosters in a bimodal vaccine regimen.

Two VaxGen vaccines have recently completed phase III trials: AIDSVAX B/B and AIDSVAX B/E (Berman *et al.*, 1999; VaxGen website: http://www.vaxgen.com). AIDSVAX B/B is a subunit vaccine made up of rgp120 from HIV-1_{MN} and HIV-1_{GNE8} (both subtype B). The AIDSVAX B/B trial took place in North America and Europe and involved 5400 volunteers at risk for HIV infection. The primary endpoint of the trial was to prevent sexual transmission of HIV-1. The vaccine did not achieve this endpoint, however there were significant reductions in HIV-1 infection among black and Asian vaccinees. The protection from infection coincided with higher levels of neutralizing antibodies observed in these two ethnic groups. AIDSVAX B/E is a similar vaccine made up of rgp120 derived from HIV-1 isolate sequences of clade B and

of clade E, which is common in Thailand where this trial took place. The primary endpoint of the trial was to prevent blood-borne HIV-1 transmission in 2546 injecting drug users. The secondary endpoint was to slow disease progression in vaccinees who became infected with HIV-1. Neither of these endpoints was achieved.

However disappointing the protective outcome of these first two phase III trials may be, they provide an enormous amount of useful information regarding immunogenicity, safety and logistics of vaccination and follow up. These vaccine trials have demonstrated a function model for phase III trials of other AIDS vaccines.

If an AIDS vaccine is to be effective it should expose conformational epitopes in the gp120 for the induction of high titre virus neutralizing antibodies to primary isolates and it should present multiple epitopes in the envelope, gag and other virus proteins to stimulate specific CTL responses with cross-clade reactivity (Ada & McElrath, 1997). A further complexity to be overcome in the vaccine design involves the presence of CTL epitopes that can be presented by the diverse MHC class I alleles found within the population to be vaccinated (McElrath *et al.*, 1997). Systems of vaccine delivery that stimulate mucosal immunisation is another area of research to be developed, as the majority of HIV infections are transmitted via this route (Lehner and Anton, 2002).

1.9 AIDS Research in Uganda

1.9.1 Preliminary Studies

At about the time when the first cases of "slim disease" were being recorded and shown to be associated with the presence of antibodies to HIV-1 (Serwadda *et al.*, 1985) the extent of HIV infection in several hospitals was examined in a number of serosurveys. These were organized mainly by local clinicians and nurses who dispatched many blood samples for analysis at the Centre for Applied Microbiology (CAMR) at Porton Down, Wiltshire, UK, thereby

initiating a prolonged collaboration between Uganda and CAMR in AIDS research. These serosurveys demonstrated the severity of the HIV epidemic in urban Uganda and along the main highways (Carswell, 1987; Hudson *et al.*, 1988; Carswell *et al.*, 1989; Berkley *et al.*, 1990) and led to the first isolations of Ugandan variants of HIV and the characterization of some of these in MRC-funded projects based at CAMR (Downing and Roff, unpublished data; Oram *et al.*, 1990, 1991). The preliminary serosurveys were followed by more systematic surveys organized by the WHO and based in the Uganda Virus Research Laboratory at Entebbe.

1.9.2 The WHO and the Uganda Virus Research Institute (UVRI)

The UVRI (Director: Dr S. Sempala) has a long history of bio-medical research and through its Department of General Virology (Head: Dr Benon Biryahawaho) the Institute collaborated with the WHO and several European and American laboratories to undertake virological and molecular studies on Ugandan variants of HIV-1. This collaboration led to the development of the capacity for large scale serosurveillance studies and in 1987 the Institute undertook a national survey to determine the extent of HIV infection in various parts of the country. This survey revealed a peak in HIV-1 infection in 20-24 year old women at 25% and a peak for men aged 25-29 years at 24%; overall women were 1.31 times more likely to be infected with HIV than men (Berkley *et al.*, 1990). However only 60% of these positives were confirmed by Western blot and the overall prevalence may have been lower. In addition to its continuing role as a primary HIV diagnostic centre, the Department has developed the facilities and expertise for the virological, serological and molecular biology techniques required for the isolation and characterization of HIV, including tissue culture, the analysis of HIV antibodies and the genotyping of viruses by heteroduplex mobility assays (HMA) of polymerase chain reaction (PCR) products from DNA extracted from leucocytes from infected persons.

Through its AIDS clinic (Head: Dr B. Tugume), UVRI provides a diagnostic service for the local population and the clinic has been a valuable source of HIV-

infected materials. The Department of General Virology has initiated collaborative projects with other laboratories, including CAMR, to provide virus isolates, virus infected cells and matched sera for collaborative studies in the UK and elsewhere. Many of the viruses and sera used in this study were produced from blood samples obtained by the Department from the UVRI clinic.

The scientific capacity of the Department is recognized world wide and in 1995 the MRC made a joint award of a project grant to UVRI and the MRC programme on AIDS in Uganda (MRCPA) with the objectives of further strengthening the virological capacity at UVRI in order to characterize Ugandan variants of HIV. A summary of the work done in this project is given at the end of the next section.

1.9.3 The Medical Research Council Programme on AIDS in Uganda (MRCPA)

In 1988 the British MRC was approached by the Ugandan government for assistance in the emerging HIV-1 epidemic. This request led to the development of the MRC Programme of Research on AIDS in Uganda based at the UVRI in Entebbe. The aims of the programme were to investigate the epidemiology and the natural history of HIV-1 infection and associated disease progression in rural Uganda. To date HIV-2 infection has not been detected in Uganda, although it is present to the south in the bordering area of north western Tanzania (Schmutzhard *et al.*, 1989).

In 1989 a general population cohort was established in a sub-county in the rural Masaka district one hundred miles south west of Entebbe (See map in Appendix II). Almost half of all adult deaths in this rural sub-county are HIV-related and 80% of young (13 - 25 years) adults' deaths are due to AIDS. This cohort was set up including about 10,000 people, half of them adults over 13 years of age. The general population cohort is surveyed annually and yields information on the prevalence and incidence of HIV-1 in the area and the effects of education and intervention studies. Recruitment of individuals to the cohort averaged 70% in the 15 neighbouring villages, but was lower in the trading centres, and follow

up averaged 62%. HIV-1 seroincidence in this area had been estimated at 1% per year in a study of nearly 8000 subjects (Mulder *et al.*, 1994). This study has now completed the tenth annual round of the survey. Age-sex standardized HIV-1 seroprevalence in this cohort over the last 4 years has declined from 7.7% to 6.4% (Whitworth *et al.*, 2002).

In 1990 the Natural History cohort in rural SW Uganda was established within the general population cohort. To date, 390 participants have been enrolled, 93 seroprevalent subjects, 86 seroincident subjects and 168 sero-negative control persons of whom 43 are of discordant relationships. The aims of this cohort are to determine the rate of progression to disease and the risk factors for progression. In the Natural History cohort there is continual recruitment of sero-negative spouses of discordant relationships, new cases of HIV-1 infection and their matched HIV-1 negative controls from the general population cohort. The Natural History cohort sees participants quarterly at the clinic where a detailed health questionnaire is filled out. A full medical examination is carried out and treatment is administered by the clinicians. Blood, faeces and urine samples are collected for laboratory investigations including tests for STDs, T cell counts and isolation of HIV-1. The HIV-1 prevalence rates in young men (aged 13 to 24) had fallen from 3.5% in 1990 to 1% in 1995 and in young women (aged 13 to 24) from 9.7% to 7% in the same time course. The overall prevalence rate of the rural study population in the Natural History cohort had stabilised at 8% (Kengeya-Kayondo *et al.*, 1995, 1996; Mulder, 1995).

The MRC ADP project grant, which terminated in September 1996, was focused on the isolation and characterization of primary isolates from cohorts in the MRCPA. One of the main objectives of the project was that the UVRI/MRC-ADP facility would act as a primary laboratory which would supply samples from infected persons for analysis in collaborating secondary laboratories in the UK and Europe. This objective was successfully achieved by collaborations with the Regional Public Health Laboratory at Cambridge, which housed one of the two MRC Repositories for HIV in the UK, the University of Edinburgh, which accommodated the other MRC Repository, the WHO International Laboratory

for Biological Standards based at the National Institute for Biological Standards & Control (NIBSC) at South Mimms, Hertfordshire and CAMR at Porton Down which also had large culture and sample collections. Of special interest in this study was the virus isolation and characterization work carried out in Dr Ulrich Desselberger's laboratory at Cambridge and by Dr Harvey Holmes at NIBSC. As shown in the Materials and Methods Section, primary isolates and sera from the Natural History cohort in rural SW Uganda were obtained from the MRC's Cambridge repository: almost all of these had been genotyped by DNA analysis of sequences from the *gag* and *env* genes. In addition, primary virus and serum samples from persons in the Entebbe/Kampala area were obtained from the WHO.

The MRC-ADP project at Entebbe had genotyped about 100 proviruses from the Natural History cohort in rural SW Uganda by DNA sequence analysis of the *gag* (p24) gene and by heteroduplex mobility assays (HMA) of the V3-V5 regions of the *env* gene. Sequence data was obtained for 73 proviruses, 41 of which were assigned to clade D, 27 to clade A and 1 to clade C: although clade B proviruses have been reported from Uganda, none were detected in this cohort. Phylogenetic analysis of the *gag* sequences revealed that there is considerable diversity within the p24 region in both clades, with a maximum divergency of about 10%. This level of divergence is probably a reflection of the long established presence of HIV in this community. More closely related sequences were obtained from sexual partners in the 4 known couples included in the study. HMA analysis of the *env* gene gave broadly similar results: out of 84 which gave unambiguous data, 32 proviruses were assigned to clade A and 52 to clade D. However, comparison of the two sets of data indicates that some of the proviruses were recombinants. Thus, for the 60 proviruses for which both DNA sequence and HMA data is available, 10 (16.7%) had *gag* and *env* gene sequences from different clades. A similar frequency of recombinant genomes has been reported for several other African countries where multiple clades of HIV are present (Cornelissen *et al.*, 1996; Kampinga *et al.*, 1997). These recombinants must have been produced during mixed infections and lead to

rapid major changes in virus genotype. The implications of a large proportion of genetic recombinants in disease progression or vaccine strategies are unknown. The Natural History cohort in rural SW Uganda offers a unique opportunity to study the interaction between a relatively well characterized group of viruses and their infected hosts.

1.10 Study Objectives

There were two main objectives in the work presented in this study:

Firstly, to develop an assay to determine the subtype or clade of the viruses which were circulating in Uganda. A simple and inexpensive assay would be invaluable in epidemiological studies including, for example, the distribution of different viral clades in different parts of a country or wider region and to show if the pattern was changing with time. At present, HIV variants are typed by molecular techniques involving the amplification of viral or proviral nucleic acid sequences by the polymerase chain reaction (PCR) followed by DNA sequencing or heteroduplex mobility assay (HMA). As these methods are very time-consuming, require sophisticated laboratory facilities and experienced staff, they are very expensive. Therefore, the objective was to develop an enzyme-linked immunosorbent assay (ELISA) which could be used with plasma or sera to distinguish between the subtypes or clades of HIV-1 thought to be circulating in Uganda at the time: in 1991 these were thought to be clades A, B, C and D.

Secondly, to study some of the virological and immunological factors that might be important in the development of a vaccine against HIV and AIDS in East Africa. As the work has progressed it has become increasingly apparent that although vaccination appears to protect monkeys from simian AIDS (Daniel *et al.*, 1992; Cranage *et al.*, 1997; Joag *et al.*, 1997), the correlates of infection are not known. Similarly, the factors (genetic, viral, immunological, environmental) responsible for the marked differences progression to disease in infected

humans are also unknown. It seems probable an effective vaccine against HIV and AIDS will have to stimulate similar protective responses in the vaccinees and it is therefore important that these factors and their interactions should be investigated both in infected persons and *in vitro*. I have studied the interaction between virus neutralizing antibodies and Ugandan viruses from the two main clades (A and D) present in Uganda with the object of correlating this data with other viral properties - chiefly viral phenotype - and with clinical data obtained from patients in the Natural History cohort in rural SW Uganda (Section 3.8). I have also produced HIV-neutralizing antibodies in laboratory animals and compared their properties with those of antibodies in human HIV-1+ antisera.
This work was started at a time when the influence of virus clade on neutralizing activities was not known. The early data was conflicting with one report that neutralization of variants from Thailand appeared to be clade specific; antibodies from persons infected with clade E viruses did not neutralize clade B viruses, and vice versa (Mascola *et al.*, 1994). Further data have indicated that many human sera had good cross-clade neutralizing activities (Trkola *et al.*, 1995; Kostrikis *et al.*, 1996; Mascola *et al.*, 1996a; Moore *et al.*, 1996). However, when this work was started this question had not been analyzed in depth and it was not known, for example, how many viruses were able to evade neutralization, whether disease progression was related to the emergence of neutralization-escape mutants.

In collaboration with the MRCPA and UVRI in Entebbe I have had access to isolates of HIV-1 and HIV-1 positive antisera and plasma from the UVRI clinic and from the Natural History cohort in rural SW Uganda. I also made two working visits to the MRC laboratories and UVRI in order to obtain follow-up samples from members of the Natural History cohort. I have used materials from both of these sources in the work described in this thesis.

The work has been organized into four main areas:

1/ Analysis/characterization of Ugandan sera using a panel of synthetic peptides corresponding to North American and African V3 loop linear epitopes.

2/ Raising of antibodies against peptides corresponding to a major neutralization epitope in the V3 region of the surface glycoprotein and the characterization of these antibodies.

3/ Isolation and phenotypic characterization of variants of HIV-1 from two regions of Uganda.

4/ Characterization of neutralizing antibodies using cell line adapted and primary isolates of HIV-1.

Finally, I have tried to relate the virological and immunological properties of some of the isolates with the clinical data obtained from the Natural History cohort in rural SW Uganda.

2. MATERIALS AND METHODS

All the materials used in the study are detailed in Appendix IV.

2.1 Viruses and matching plasma/sera

Some primary isolates and many plasma, designated with "U" numbers, were obtained from the CAMR repository of African isolates and plasma samples. These samples were prepared from blood samples received at CAMR in 1985/6 from Dr Wilson Carswell (Mulago Hospital) who obtained them from AIDS patients attending various clinics and hospitals, mostly in the Kampala area of Uganda.

Samples of sera/plasma were also obtained by Dr Hanny Friesen and her colleagues in Save The Children from sero-positive asymptomatic mothers attending ante-natal clinics in Kampala in 1985/7. These were also dispatched directly to CAMR.

Additional plasma/serum samples from 42 AIDS patients and 93 asymptomatic persons, taken mostly in the UVRI clinic between 1990 and 1992, were obtained directly from the Department of General Virology at UVRI (Department Head: Dr Benon Biryahawaho). Matching samples of leucocytes, frozen in liquid nitrogen, were also obtained for many of these samples and I made primary isolates from some of these, as described in Section 2.8.2., to give a group of twelve paired samples of antiserum and virus from HIV-1+ asymptomatic individuals. A further 2 matched primary virus/plasma samples were made and identical samples of these were dispatched to the World Health Organization and may obtained through Dr Harvey Holmes at the National Institute for Biological Standards and Control. These isolates and their genotypic and phenotypic properties are described in the Results Sections 3.3 and 3.4.

For most of these samples, the dates of sero-conversion were unknown, there was very limited clinical data and it was not possible to obtain follow-up samples at later dates. However, from the beginning of 1994, well characterized

samples became available from actively managed cohorts within the MRC Programme on AIDS in Uganda.

Paired virus and plasma samples from the sero-positive participants in the MRCPA Natural History cohort were obtained from the MRC AIDS Reagent Programme (Director: Dr Harvey C. Holmes). The viruses had been isolated in one of the Programme's collaborating laboratories - the Public Health Laboratory at Cambridge (Director: Dr Ulrich Desselberger) - within the MRC-funded Repository for HIV. The corresponding proviruses had been subtyped by DNA sequencing of their *gag* and *env* genes by Catriona Baker, working in the same laboratory.

Natural History cohort Incident Virus/Plasma Samples:

Sample number	Age	Sex	Sero-conversion	Isolation date
3004	27	M	21.04.92	18.11.93 (19)
3011	35	M	09.09.93	18.01.94 (16)
3012	32	M	24.03.91	18.01.94 (34)
3013	29	M	08.09.92	18.01.94 (16)
3018	18	F	13.06.91	18.01.94 (31)
3021	44	F	02.07.91	22.02.94 (31)
3025	39	M	09.01.92	22.02.94 (25)
3028	36	F	09.04.91	22.02.94 (34)
3029	53	F	09.12.91	22.02.94 (26)
3032	27	F	23.04.91	13.04.94 (36)
3046	21	F	19.11.91	26.05.94 (30)
3048	51	M	27.01.93	26.05.94 (16)
3049	32	M	01.06.92	26.05.94 (23)
3052	40	M	06.02.93	13.07.94 (17)
3053	55	M	17.10.93	13.07.94 (9)

Numbers in brackets = months since seroconversion. Seroconversion dates were estimated from the time of the first positive and the time of last negative results.

I made additional isolates, as described in Section 3.5.2.2, from blood samples obtained directly from the Natural History cohort in rural SW Uganda by Dr Dilys Morgan of the MRCPA, who also supplied sequential serum samples from some of the persons listed above.

Before use all sera were heat inactivated at 56°C for 1 hour; this destroyed any virus present thus allowing their use outside of containment facilities and also destroyed any complement components.

2.2 Peptides

The following peptides for use in antibody binding assays, competitive inhibition assays and adsorption assays were based on published V3 loop sequences (Gurgo *et al.*, 1988; Oram *et al.*, 1991; Albert *et al.*, 1992; Myers *et al.*, 1993; Bruce *et al.*, 1994; Robertson *et al.*, 1995). These peptides were supplied by the AIDS Reagent Project of the Medical Research Council or purchased from Cambridge Research Biochemicals, Northwich, UK. The countries of origin of the proviruses are shown in parentheses and their genetic subtypes in square brackets:

MN (USA)	RKRIHIGPGRAFYTTKN	[B]
MAL (ZAIRE)	RRGIHFGPGQALYTTG	[A/D]
Z6 (ZAIRE)	RQSTPIGLGQALYTTRGRT	[D]
Z3 (ZAIRE)	RQSIRIGPGKVFYAKGG	[U]
U1685 (UGANDA)	IQRTSIGSGQALYTTR	[D]
U2999 (UGANDA)	IQRTHIGTGQALHTTR	[D]
U31 (UGANDA)	RQRTPIGLGQALYTTK	[D]

The following consensus peptides spanning the V3 loop apex were based on consensus sequence data for subtype A and D Ugandan proviruses (Albert *et al.*, 1992; Bruce *et al.*, 1994):

UG A apex	RKSVHIGPGQAFYATG
UG D apex	RQSTHIGPGQALYTTN

These and the following peptides, which were based on the same published data (Gurgo *et al.*, 1988; Albert *et al.*, 1992; Bruce *et al.*, 1994), were synthesized by Dr Cliff Shone, CAMR, Porton Down.

MN	V3 loop apex	HIGPGRAF
UG D	V3 loop apex	HIGPGQAL
UG D	NH_3 terminus of V3 loop	CTRPYNNTRQSTHI
UG D	COOH terminus of V3 loop	GQALYTTNIIGDIRQAHC

For the immunization of rabbits, the following full length V3 loop peptides were obtained from the AIDS Reagent Project of the MRC (Dr Harvey Holmes). This part of the project was funded by the European Vaccine for AIDS (Project EVA) and the peptides were synthesised by Dr M.S.Munns (Washington Singer Laboratories, University of Exeter): The amino acid sequences were confirmed by nuclear magnetic resonance by Dr Robin Wait (CAMR, Porton Down).

UG A:

Ac-**CAA**CTRPNNNTRKSVHIGPGQTFYATGEIIGDIRQAHC-NH_2

UG D:

Ac-**KAA**CTRPYNNTRQSTHIGPGQALYTTNIIGDIRQAHC-NH_2

U31:

Ac-**EAA**CTRPYYNIRQRTPIGLGQALYTTKGRGTTKVIGQAHC-NH_2

MN:

Ac-**EAA**CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC-NH_2

Additional residues were added to the N-terminal cysteine residue of the V3 loop, shown in bold in order to couple the peptides to a protein carrier (see below).

2.3 Antibody Binding Assays

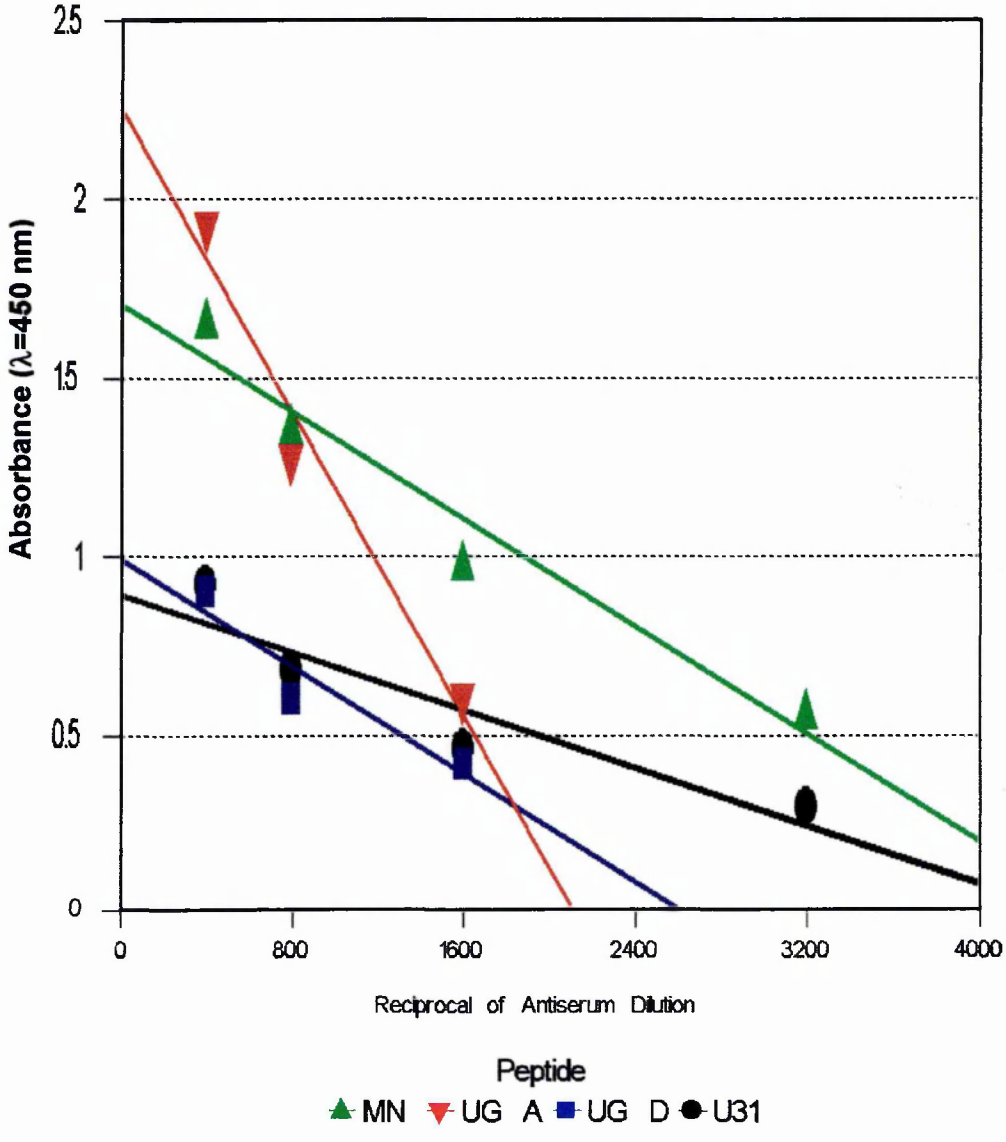
Peptides were dissolved in water at 2µg/ml and 50µl (100ng) dried overnight in the wells of 96 well plates (Nunc). The plates were washed 5 times with wash solution - phosphate buffered saline (PBS; 8.0g/l NaCl, 1.21g/l K_2HPO_4, 0.34g/l

KH_2PO_4, pH 7.3) containing 0.1% Tween 20 - and blocked with wash solution containing 10% calf serum for 1hr. at 37°C. Sera were diluted in blocking solution and 100µl added to coated and uncoated (control) wells and incubated for 2 hr. at room temperature. The plates were washed and reacted with 100µl of conjugate for 1hr. 45min at room temperature to detect binding of the antibodies to the peptides; anti-human IgG-horse radish peroxidase (Sigma) was used for human sera and anti-rabbit IgG-horse radish peroxidase (Jackson ImmunoResearch Laboratories, Inc.) was used for rabbit sera. The conjugated antibodies were diluted in wash solution containing 10% calf serum at the manufacturers' recommended dilutions, which varied from batch to batch. The plates were washed 5 times with wash solution and the bound antibody determined by reaction with 100µl of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) solution - one TMB tablet (Sigma) dissolved in 10ml of buffer (0.1M citric acid, 0.2M dibasic sodium phosphate, pH 5.0) plus 2µl of 30% H_2O_2. After 30 min. the reaction was stopped by the addition of 50µl of 2M H_2SO_4. The absorbance was read at λ=450nm (Devash *et al.*, 1990).

Most sera were analyzed at a single dilution, usually 1:200, and those giving absorbency readings at λ=450nm greater than twice that obtained in the absence of peptide were scored positive. Some sera were assayed at serial two-fold dilutions and the readings used to calculate a titre arbitrarily defined as the dilution that corresponded to an absorbance value of 0.5 after subtraction of the negative control. To control the effect of day-by-day assay variations, a reference serum (U 3529), which reacted with all the peptides used in the study, was included in each set of assays. Figure 8 shows the titration by two-fold serial dilution of antiserum U3529 as an example of a graph from which the serum titre at absorbance value 0.5 was measured.

Figure 8. Shows the titration by antibody binding assay of two-fold serial dilutions of antiserum U3529 as an example of a graph from which the serum titre at absorbance value 0.5 was measured. The graph shows the linear relationship between the absorbance (λ=450nm) and the antiserum dilution in the assay.

Figure 8


2.5
2
1.5
1
0.5
0
Absorbance (λ=450 nm)
0
800
1600
2400
3200
4000
Reciprocal of Antiserum Dilution
Peptide
MN
UG A
UG D
U31

Appendix VI shows a comparison of serum titres at the absorbance value of 0.5 and the absorbancy readings at 1:200 serum dilution. A number of duplicate readings are also shown to demonstrate the consistency of the assays. To justify the use of both types of measurements to establish positive or negative reactivity of a serum with a peptide it was necessary to compare some data. I considered that a high reading at 1:200 should reflect a high titre at 0.5 absorbance if these two types of reading were to be used in this thesis. In view of the good semi-quantitative correlation between the two methods, shown graphically in Appendix VI, I felt justified in using the much more rapid single serum dilution method for analysis of a large number of samples. The absorbance readings at 1:200 serum dilutions enabled the reactivity of many sera to be assessed by avoiding repeated experiments with serial dilutions of each serum. All assays were carried out using duplicate samples and all were repeated to confirm the results. No direct quantitative comparisons were made between these two types of measurements of serum reactivity with peptides.

Larger amounts of peptides gave no significant increase in the specific signals, indicating that the well surfaces were saturated by 100ng of peptide.

The mean and standard deviations were calculated for titrations of the reference serum with each peptide and results with a probability of greater than 0.05 of variation outside the mean value were discarded.

The data obtained from these antibody binding assays were grouped and presented in Table 4 in section 3.2.3. The tabulated data was subjected to Chi squared analysis. The null hypothesis was rejected if the probability of an error was less than 5%.

2.4 Competitive Inhibition Assays

These were performed by pre-incubating 100µl of a dilution of antiserum with 100µl of serial two-fold dilutions of peptides in blocking solution for 2 hr. at room temperature; the mixtures were then added to antigen coated or un-coated wells in antibody-binding assays, as above (Wolfs *et al.*, 1991).

2.5 Adsorption of Antibodies to Sepharose-Peptide Columns

Activated CH-Sepharose 4B (Pharmacia) is formed by esterification of the carboxyl groups in the agarose polymer with *N*-hydroxysuccinimide; this active ester is able to form covalent bonds with primary amino groups.

Peptides were coupled to activated CH-Sepharose 4B by adding 1mg of peptide dissolved in 0.1M sodium bicarbonate buffer, pH8.0 to 1ml of activated Sepharose for 1 hr. at room temperature. The peptide-Sepharose mixture was then transferred to a small column, washed with blocking solution and 0.5ml of a 1 in 20 dilution of antiserum in blocking solution was applied to the column. After 1 hr. at room temperature, non-adsorbed antibodies were eluted with 2ml of blocking solution and assayed as described above.

2.6 Coupling of Peptides to Carrier Protein

Coupling of the V3 loop peptides to keyhole limpet haemocyanin (KLH) was achieved through the side-chain of terminal amino acid residues added to the N-terminal amino acid of the V3 loop peptide. To facilitate specific rather than random couplings, the peptides were synthesized with additional amino acid residues, as noted above. As the peptides had different amino acid sequences it was necessary to use three methods to achieve specific coupling of the carrier protein to N terminal residues of the peptides. The methods were:

2.6.1 Glutaraldehyde Coupling, which couples via the free amino group in the N-terminal lysine residues, was used for the UG D peptide which did not contain any lysine residues in the V3 loop.

1-2mg of peptide was dissolved in a solution of 4mg/ml KLH in 0.01M sodium phosphate (pH 7.4) and 10µl 25% (v/v) glutaraldehyde was added in 2.5µl aliquots. The mixture was stirred at 4°C for 60 min and then for a further 60 min after the addition of 0.5mg of sodium borohydride (Briand *et al.*, 1985). The conjugate was purified by dialysis.

2.6.2 Carbodiimide Coupling, which couples via the free carboxyl group of glutamic or aspartic acids (Goodfriend *et al.*, 1964) was used with the MN and U31 peptides, both of which contained lysine but not acidic amino acid residues within their V3 loops.

0.5ml of an aqueous solution (4mg/ml) of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) was added to 0.5ml of water containing 2mg of peptide. The pH was adjusted to 4.5 - 5.1 and the solution was stirred for 5 mins. 5mg of KLH dissolved in 0.5ml of water, pH 4.5 to 5.1, was added to the peptide-EDC mixture and stirred overnight at room temperature. The conjugate was purified by gel filtration through a 30 by 1.6 cm column of Sephadex G-50 (fine: Pharmacia) equilibrated with 150mM NaCl at 4^0C and fractions containing the conjugate were detected spectrophotometrically at λ=280.

2.6.3 Coupling with maleimidobenzoyl-N-hydroxysuccinimide (MBS): this method couples via disulphide bridges between cysteine residues and was used for the UG A peptide, which contained both lysine and glutamic acid residues within the V3 loop.

Maleimidobenzoyl-KLH (KLH-MB) was produced by adding 10µl aliquots of MBS (1mg in 50µl dimethyl formamide) to 250µl KLH (4mg/ml in 0.01M sodium phosphate, pH 7.4). Unreacted MBS was removed after 30 min. by filtering through a PD 10 column (a Sephadex G-25 Medium column, Pharmacia) equilibrated with 0.05M sodium phosphate (pH 6.0): fractions containing the conjugate were detected spectrophotometrically at λ=280. The MB-KLH fraction was then titrated to pH 7.5 with 2M NaOH and mixed for 2-3 hours with 40-100 fold excess of peptide (Green *et al.*, 1982). Peptide concentration was estimated from the amount of free thiol using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) assay (Riddles *et al.*, 1979). After incubation the solution was typically turbid.

2.7 Immunization of Rabbits

Sixteen New Zealand White rabbits were used in the peptide inoculation programme. All of the rabbits were pre-bled and their sera were assayed by the antibody binding assay and found to have no V3 loop specific IgG reactivity. Four rabbits were inoculated with each of the peptides: two with conjugated peptide-KLH and two with uncoupled peptide as shown in Table 12.a in the results Section 3.7.1. The vaccination programme consisted of subcutaneous inoculations spaced four weeks apart; two inoculations of 100ng of the specified conjugated or unconjugated peptide mixed 1:1 with Freund's incomplete adjuvant, followed by four inoculations of 100μg of the peptide or peptide conjugate containing the same amount of peptide mixed 1:1 with Freund's incomplete adjuvant[1]. Two weeks after each of the 100μg inoculations a blood sample was taken from the marginal vein of each rabbit's ear. These blood samples were allowed to clot for 30 min at 37°C. The clots were reduced overnight at 4°C the sera were then removed from the clot with a pastette. The sera were assayed for V3 loop specific IgG activity by the antibody binding assay described above (data are shown in the Results Section).

2.8 Preparation of Peripheral Blood Mononuclear Cells

2.8.1 Preparation of Feeder Cells for Co-cultivation Experiments

Concentrated buffy coat cells obtained from the Blood Transfusion Service typically containing about 8 x 10^8 cells, mostly white cells in about 100ml of plasma were diluted 1:1 in Dutch modified RPMI 1640 cell medium (ICN Flow) containing 10% foetal calf serum (RPMI/FCS): The suspension was centrifuged at 200g for 10 min. at 25°C and the diluted plasma was discarded. The sedimented cells were resuspended in the same medium and 35ml gently layered onto 15ml of Ficoll-Paque (Pharmacia) in 50ml centrifuge tubes, which were centrifuged at 250g for 30 min. at 25°C. Using a wide bore pastette, the

[1] Personal correspondence from Dr Han Huisman, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, PO Box 9190, 1006 AD Amsterdam.

lymphocytes were taken off from the layer above the red blood cells, resuspended in RPMI/FCS and washed twice by centrifugation at 250g for 10 min (Bϕyum, 1968). For immediate use in virus cultivation experiments, the PBMC were resuspended to 5 x 10^5 cells/ml in RPMI/FCS containing 100IU/ml Kanamycin (Sigma). After 6 hrs, the culture was stimulated with phytohaemagglutinin (PHA) at 1μg/ml and incubated at 37°C for 4 days. The cells were centrifuged at 200g for 15 min and then resuspended in fresh medium containing 10 IU/ml interleukin-2 (IL-2) obtained from the MRC-ARP.

For long-term storage, the freshly prepared PBMC were resuspended in 90% FCS plus 10% dimethyl sulfoxide (DMSO: Sigma) to give approximately 5 x 10^6 cells/ml and 1ml aliquots frozen (-2°C/min) before transfer to the gas phase of liquid nitrogen[2]. These stocks were then resuscitated for use in virus cultivation and neutralization experiments.

2.8.2 Preparation of PBMC from Infected Persons

Five millilitre blood samples were collected from infected persons in 7 ml vacuum tubes containing EDTA (Becton Dickinson Vacutaner Systems). The blood samples were handled in the Category II containment laboratory at UVRI in Entebbe. They were transferred into 15ml plastic tubes and were centrifuged in sealed buckets at 250g for 5 min at 25°C and the plasma were carefully collected from the surface and stored at -80°C for experimental use. The cells were diluted in RPMI/FCS to a volume of 10ml and the whole suspension gently layered on 4ml of Ficoll-Paque in 15 ml centrifuge tubes. These were centrifuged at 400g for 30 min at 25°C. Using a wide bore pastette, the lymphocytes were taken off from the layer above the red blood cells, resuspended in 10ml of RPMI/FCS and washed twice by centrifugation at 250g for 15 min. The lymphocytes were resuspended in FCS plus DMSO and stored

[2] Stocks of PBMC were PHA stimulated after storage prior to use in the virus coculture and neutralization experiments described below, this enabled the use of the same unstimulated PBMC stocks in other experiments.

in liquid nitrogen, as above, before transfer, with the matching plasma samples, to CAMR in a "dry shipper" containing liquid nitrogen.

For virus isolation, the infected PBMC were cocultivated with feeder cells in a category III containment facility at CAMR, as described in the following section.

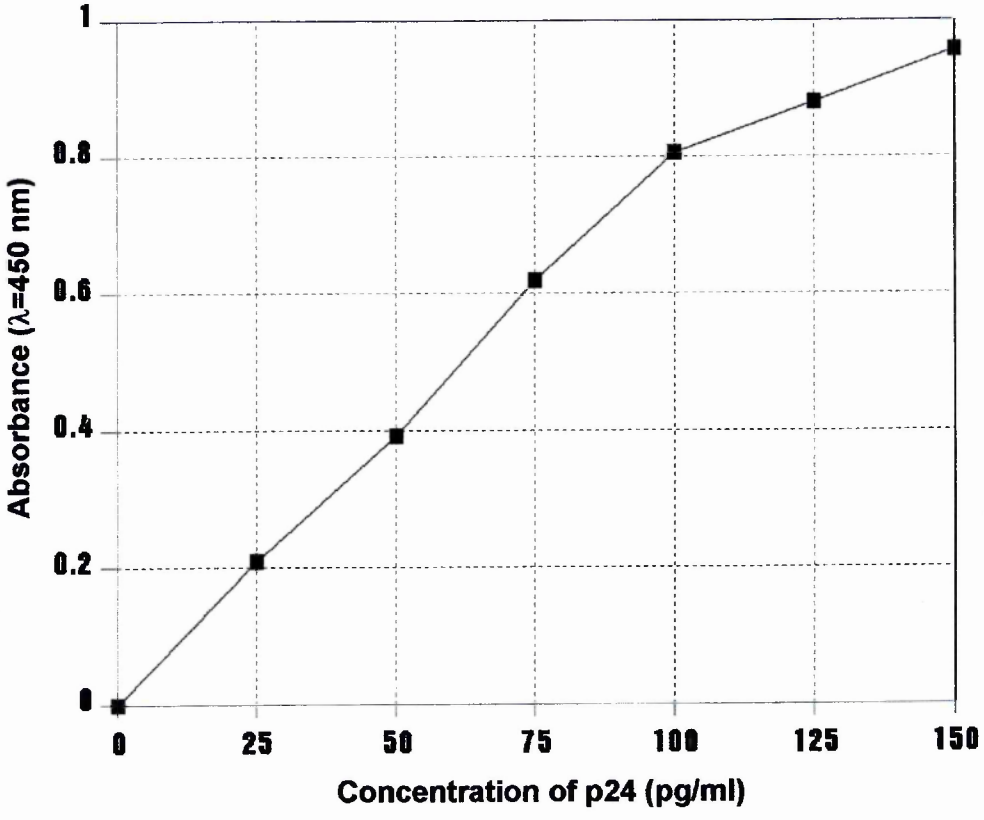
2.9 Virus Isolation

For virus isolation, the infected PBMC were co-cultivated with feeder cells in a category III containment facility at CAMR. This was done by co-cultivating 1-2 x 10^6 patient PBMC with 1.5-2 x 10^6 PHA-stimulated PBMC/ml in 10mls of RPMI/FCS containing 10 IU/ml recombinant IL-2 (RPMI/FCS/IL-2) in $25cm^3$ flasks. The cultures were re-fed every 3 or 4 days with RPMI/FCS/IL-2; once a week, 0.5-1 x 10^6 PHA-stimulated PBMC were added. The cultures were examined daily by light microscopy for the development of a cytopathic effect. The development of syncytia indicated virus growth in the culture. The supernatant was removed once a week to monitor HIV p24 antigen (p24 Ag) production for evidence of virus growth.

Before testing for p24 Ag, the virus supernatants were treated with $1/10^{th}$ volume of 1% Empigen and heat inactivated at 56°C for 30 min. The vials were then sprayed with 70% alcohol and removed from the category III laboratory to the serology laboratory. The Coulter p24 antigen assay kit was used for these experiments as recommended by the manufacturer (Popovic *et al.*, 1984; Burke *et al.*, 1990). To calculate the p24 concentration in virus cultures, a calibration curve for absorbance at 450nm against p24 Ag concentration was plotted for each assay using the antigen provided in the kit manufacturer's kit. An example of a p24 calibration curve is shown in Figure 9. Several attempts were made to prepare "in house" p24 assay kits, using the components and methods recommended by the MRC-ADP. However, I found that the sensitivity of the "in

Figure 9. Calibration curve obtained for the absorbance (λ=450nm) versus concentration of p24 antigen standard, measured using the Coulter p24 antigen assay kit.

Figure 9


1
0.8
0.6
0.4
0.2
0
Absorbance (λ=450 nm)
0
25
50
75
100
125
150
Concentration of p24 (pg/ml)

house" assay was much lower than that of the Coulter kit and was not suitable for measuring the low levels of p24 produced in microtitre plate cultures of many primary isolates.

I found that primary cultures containing less than 100pg/ml of p24 antigen were unsuitable for use in virus neutralization experiments; after inhibition by antiserum they produced amounts of p24 antigen which were below the sensitivity of the Coulter p24 antigen assay kit.

Isolation attempts were terminated on the 28th day. In positive cultures suitable for virus neutralization experiments, 1ml aliquots of the supernatants and of cells resuspended in 90% FCS plus 10% DMSO were transferred to 1.8ml cryotubes (Nunc) and stored in the gas phase of liquid nitrogen.

2.10 Virus Expansion

Virus expansion was carried out by incubating 2 x 10^6 infected cells with 1.5-2 x 10^6 PHA (1μg/ml) -stimulated PBMC/ml in 25 cm^3 tissue culture flasks (Corning) and the volume made up to 10 ml with RPMI/FCS with 10 IU/ml IL-2: 2 flasks were set up for each isolate and a culture containing 1.5-2 x 10^7 uninfected PBMC in 10ml of RPMI/FCS with IL-2 was kept as a control. The medium was changed twice weekly and 2-3 x 10^6 PHA-stimulated cells were added once a week (Folks *et al.*, 1986).

Isolates were adapted to grow in several T cell lines (MT-2, C8166, JM and H9) and the monocyte line U937 (Schwenk and Schneider, 1975; Sundstrom and Nilsson, 1976; Miyoshi *et al.*, 1981; Salahuddin *et al.*, 1983; Popovic *et al.*, 1984). Virus supernatants from infected PBMC cultures were incubated with the cell line (5 x 10^5 cells/ml) in 10ml of RPMI plus 10% FCS and 100 IU/ml kanamycin (Popovic *et al.*, 1984) in 25cm^3 flasks. These cultures were incubated at 37°C and 5ml of the supernatant was replaced with fresh medium every 4 days. The cultures were examined daily by light microscopy for the development of a cytopathic effect. The supernatant was removed once a week to monitor HIV

p24 antigen (p24 Ag) production for evidence of virus growth. Virus expansions were terminated when the p24 level reached 100pg/ml or on the 28th day.
Virus stocks were aliquoted into 1.8ml cryotubes and stored in the gas phase of liquid nitrogen as described above.

2.11 Virus Titration

In order to obtain known titres of the isolated virus stock for use in the neutralization studies, virus titrations were carried out and the results expressed as end-point titres (EPTs), i.e. the limiting dilution at which virus growth produced 100pg/ml of p24.
The isolated virus stock supernatant was diluted in RPMI/FCS to give six ten-fold dilutions from 1:10 to $1:10^6$. A PHA-stimulated culture of PBMC was diluted in RPMI/FCS to give 1×10^6 cells/ml and 100µl dispensed into the wells of a 96-well round bottomed plate (Corning), followed by the addition of 100µl of the virus dilutions (3 replicates per dilution) to each well. The plates were incubated at 37°C in pods gassed with 5% CO_2 in air. The culture medium was changed on days 1 and 3, and a 200µl Empigen-lysed sample from each well was analyzed for p24 antigen using the Coulter p24 assay kit on day 7.
From the results of these titrations the EPT that produced 100pg/ml of p24 was determined for each virus stock grown in a particular batch of PBMC. Virus titrations that grew but produced less p24 than 100pg/ml were considered "negative" in this assessment of EPTs. I used this threshold value, because virus titrations that produced much lower p24 concentrations did not produce enough virus growth for detection by Coulter p24 antigen assay kit in the neutralization experiments after inhibition by antisera. I used the same threshold value for all of the isolates to ensure that there was a sufficiently high level of virus growth in all the neutralization experiments. The virus titrations also allowed me to use a standard level of virus infectivity in the neutralization experiments (Whalley *et al.*, 1991). The same batch of PBMC was used for both virus titration and neutralization of a particular virus stock as it has been observed that different PBMC may affect the virus growth (Folks *et al.*, 1986).

Titration of viruses was also carried out on cell lines in order to determine the EPT that produced 100pg/ml of p24 for each virus stock for each cell line in which it was grown. Virus stock supernatant from the expanded cell line culture was diluted in RPMI/FCS and 100µl of each dilution (3 replicates per dilution) was added to 100µl of the same cell line (5 x 10^5 cells/ml) in a 96-well round bottomed plate (Corning). The plates were incubated at 37°C in pods gassed with 5% CO_2 in air. The culture medium was changed on days 1 and 3, and on day 7 a 200µl Empigen-lysed sample from each well was analyzed for p24 antigen using the Coulter p24 assay kit. A titration was considered positive if the amount of p24 antigen in the supernatant was 100pg/ml or more and from these results the EPT that produced 100pg/ml of p24 was determined for each virus stock for each cell line in which it was grown.

2.12 Back-Adaption of HIV-1_{MN} to PBMC and adaption to cell lines

The cell line adapted isolate HIV-1_{MN} (Gallo *et al.*, 1984) was obtained as chronically infected H9 cells from MRC-ADP. The culture was expanded by co-cultivation with uninfected H9 cells at a ratio of 1:4, in 25cm^3 flasks. The supernatant from the culture was frozen in 1ml cryotubes and stored in gas phase liquid nitrogen. The cell line adapted MN isolate was back-adapted to PBMC by infecting 10ml of 1 x 10^6 PBMC/ml with the supernatant from the HIV-1_{MN} infected H9 cell culture in 15ml of RPMI/FCS in 25 cm^3 flasks. The culture was stimulated with IL-2 every 4 days and fresh PHA-stimulated PBMC were added to the culture weekly. The culture was maintained for 28 days and assayed for p24 antigen production every week. Multiple passages were required to obtain an end-point titre of 10^3 EPT/ml for the back-adapted HIV-1_{MN}.

HIV-1_{MN} was also adapted to grow in the C8166 and MT-2 T cell lines. To achieve this, a 10ml culture (5 x 10^5 cells/ml) was infected with 5ml of supernatant from the HIV-1_{MN} infected H9 culture in 25cm^3 flasks and

incubated at 37°C. Every 4 days, 5ml of the culture supernatant was replaced with 5ml of fresh RPMI plus 10% FCS (Popovic *et al.*, 1984) and 5 x 10^5 cells were added weekly. The culture was assayed weekly for p24 antigen production and was maintained for 28 days. End-point titres of 10^4 EPT/ml were obtained for HIV-1_{MN} in C8166 and MT-2 cells.

2.13 Virus Phenotyping

Following their isolation from infected blood samples co-cultured with PBMC, the phenotypes of the primary isolates were determined from their abilities to grow and produce syncytia in cultures of a number of cell lines: MT-2, C8166, JM, H9 and U937.

The method used to grow these isolates in the different cell lines is described in Section 2.10. The cultures were examined by microscopy once a day for the development of syncytia and once a week for the production of p24 Ag. Those developing syncytia were classed as syncytial inducing (SI) and those which produced p24 Ag in the absence of syncytia were classed as non syncytial inducing (NSI).

Virus isolates HIV-$1_{UG92001}$ and HIV-$1_{UG93070}$ were shipped from Uganda and isolated at CAMR as described above, but identical samples were sent to the WHO therefore they were labelled using the WHO system.

2.14 Virus Neutralization Assays

Using low-binding 96 well plates, HIV-1 positive Ugandan plasma/sera or rabbit antisera raised against V3 loop peptides were serially diluted twofold from 1:10 to 1:320 or from 1:20 to 1:640 in RPMI/FCS to leave 100µl of dilution in the wells. Virus was diluted in RPMI/FCS to 100 EPTs/ml and 100µl was added to the diluted sera and the reaction mixtures were then incubated for 1 hr. at 37°C. 100µl of the virus/serum (or plasma) mixture were then added to 100ul of a 4-day PHA-stimulated culture of PBMC (diluted to 5 x 10^5 cells/ml in RPMI/FCS/2xIL2) contained in round bottomed 96 well plates (Corning). The plates were incubated at 37°C in pods gassed with 5% CO_2 in air and after 6-24

hr. the medium was changed by replacing 150µl with fresh medium (RPMI/FCS/IL-2). This step was repeated on day 3 and day 5 (Vujcic *et al.*, 1990; Broliden *et al.*, 1991). This wash procedure ensured that there was a massive dilution of any unbound antibodies in the assay system after the initial antibody/virus reaction. Seven days after virus infection, 10µl of 1% Empigen (Calbiochem) was added to each well and the plates were heated at 56°C for 1 hr.: the inactivated samples were then assayed for p24 by the Coulter p24 antigen assay kit. Neutralization titres for the antisera against each viral isolate were calculated from the reduction in p24 antigen production compared with that produced in the HIV-negative human plasma control (Albert *et al.*, 1990) or the rabbit pre-bleed serum control.

All dilutions were duplicated for each assay, and each assay was repeated to verify the results: All the observations shown in the results section were reproducible. Where comparisons were to be drawn directly between sequential virus samples these neutralization assays were always part of the same experiment, were always carried out in duplicate and the assays were repeated together. Controls included wells containing medium but no plasma/sera and wells containing HIV-negative control plasma. Negative sera used included human sera of African origin and British origin; virus growth was not inhibited by any of the negative sera used. Control sera used in the assays of the rabbit sera raised to V3 loop peptides was of rabbit origin, both pre-bleed sera and rabbit sera after inoculation with adjuvant only and adjuvant and uncoupled KLH.

An example of a neutralization curve is shown in figure 14, the neutralization titre of a plasma/serum was defined as the reciprocal of the dilution which reduced p24 production by 75% (as discussed in section 3.5). Neutralization titres towards some viral isolates were also measured for antiserum GB8 raised against a British isolate, and a monoclonal antibody raised against the V3 loop apex of HIV-1_{MN} (Gorny *et al.*, 1991).

Some viruses were also neutralized on MT-2 cells. A 4-day MT-2 cell culture was diluted to 5 x 10^5 cells/ml in RPMI with 10% FCS and kanamycin 100 IU/ml. Virus neutralization on MT-2 cells was carried out using the same method and stipulations as described above for virus neutralization on PBMCs.

3. RESULTS

3.1 Serological Subtyping of Viral Isolates

One of the main objectives of this study was to develop an assay to determine the subtype, or clade, of the viruses which were circulating in Uganda so that it could be used in epidemiological studies including, for example, the distribution of viral subtypes in different parts of Uganda. The approach adopted was to develop a serological assay which utilized peptides corresponding to the V3 loop regions of viruses from different subtypes: this region had been found to show considerable sequence variation between different virus isolates (Wahlberg *et al.*, 1991; Cheingsong-Popov *et al.*, 1992) and to induce a very strong serological response in many infected persons (Neurath & Strick, 1990; Arendrup *et al.*, 1993; Montefiori *et al.*, 1993). However, at the start of the study peptide based assays for HIV had not been developed or evaluated. Therefore, once the assay had been developed, as described in Section 2.3, the next objective was to test the specificity of the assay by correlating data obtained from antibody binding assays with V3 loop sequence data from autologous proviruses.

Autologous sera corresponding to the 9 virus isolates and 5 proviruses of known V3 loop sequence were tested against V3 loop apex peptides encoded by one North American (HIV-1_{MN}), 2 Zairian (HIV-1_{Z3}, HIV-1_{MAL}), 2 Ugandan (HIV-1_{U1685}, HIV-1_{U31}) isolates and 2 apex peptides corresponding to Ugandan consensus sequences (UG A and UG D). The sera and proviral DNA sequences listed with "U" prefixes were obtained from viruses isolated from AIDS patients in 1985/6 (Oram *et al.*, 1991) and those with "ACP" prefixes were obtained from AIDS patients sampled in 1990/2 (Bruce *et al.*, 1994).

The data shown in Table 1 shows that all the 1985/6 sera reacted strongly with 3 peptides - MN, U1685 and U31 (except for U2999 which did not react with the U1685 peptide): most of the sera also reacted with the apex UG A and UG D

TABLE 1. BINDING OF ANTIBODIES TO V3 LOOP SPECIFIC PEPTIDES.

SERA (SUBTYPE) V3 LOOP APEX SEQUENCE*	SAMPLE DATE	PEPTIDES USED IN ASSAYS / SUBTYPE (COUNTRY OF ORIGIN) SEQUENCE						
		MN / B (USA) RKRIHIGPGRAFYTTKN	Z3 / U (ZAIRE) RQSIRIGPGKVFYAKGG	MAL / A/D (ZAIRE) RRGIHFGPGQALYTTG	U1685 / D (UGANDA) IQRTSIGSGQALYTTR	U31 / D (UGANDA) RQRTPIGLGQALYTTK	UG D / D (UGANDA) RQSTHIGPGQALYTTN	UG A / A (UGANDA) RKSVHIGPGQAFYATG
U455 (A) R-YS--S-QT-A-GK	1986	2000	-	-	8000	6000	-	375
U4133 (D) I-T---S-Q-Y-R-N	1986	5000	-	-	3000	4000	4000	-
U1685 (D) Q-TS--S-Q-L---R	1986	6000	-	2000	10000	8000	6400	800
U653 (D) SIRR-----WQT-YT	1986	4000	-	-	8000	5000	4800	500
U462 (D) R-TS--Q-Q-Y---N	1986	60000	4000	12000	12000	3000	1600	2900
U2999 (D) Q-T---T-Q-LH--R	1986	20000	-	10000	-	3000	5500	-
U4132 (D) Q-TS--Q---L----	1986	20000	-	1000	5000	5000	-	-
U1665 (D) Q-TP--L-Q------	1986	5000	8000	8000	5000	4000	1100	1000
U5055 (D) QSTP--L-Q-L---R	1986	20000	1500	3000	1000	2000	10000	2000
ACP37024 (D) QST-----H-LF--N	1992	1000	150	200	-	-	1000	200
ACP37025 (A) --S-R----QT--A-	1992	4500	4500	-	-	-	-	400
ACP37026 (A) --SV-----Q---A-	1992	45000	6000	700	2000	2000	7000	7500
ACP37027 -QG--------YI-E	1992	3200	1700	150	-	-	300	-
ACP38051 --SVR----Q---A-	1992	1800	900	-	-	-	-	-

Titres are reciprocals of serum dilutions giving an EIA reading of 0.5 OD units. - = titre < 100. * As a variation from HIV-1_{MN}.

consensus peptides. Six of the nine 1985/6 sera reacted with the MAL peptide, but only two reacted with the Z3 peptide.

The five 1990/2 sera showed a more varied response to the peptides. Although 4 out of 5 of these sera reacted strongly, and one weakly, with the MN and Z3 peptides and 3 reacted with the UG A and UG D apex peptides, only serum ACP 37026 reacted with the U31 peptide.

There was no obvious correlation between the V3 loop sequence of a provirus and the specificity of the autologous serum. Thus, serum U5055 reacted even more strongly with the heterologous MN peptide (RKRIHIGPGRAFYTTKN) than with peptides encoded by the Ugandan isolates U31 and U1685 which had very similar V3 loop sequences to the U5055 provirus (RQSTPIGLGQALYTTR). Moreover, serum U2999 reacted with the MN peptide but not with the U2999 peptide (IQRTHIGTGQALHTTR) - data not shown.

In a further set of experiments 7 antisera from HIV-1 seropositive asymptomatic individuals bled at the UVRI clinic in Entebbe and 1 antiserum from an AIDS patient (U1685) and 15 from HIV-1 seropositive individuals attending the clinic in rural SW Uganda were assayed using the whole V3 loop peptides of HIV-1 isolates MN and U31 from the USA and Uganda, respectively, and the two consensus peptides UG A and UG D. These results are shown in Table 2 which also gives the genetic subtype of these isolates previously determined by *gag* and *env* gene sequence analysis (unpublished data from CB Bruce and JD Oram and C Baker, M Phil thesis, University of Cambridge). One of the subtype D sera did not react with any of the 4 peptides and another subtype D serum reacted with only the MN (subtype B) peptide. Six of the sera, one subtype A and 5 subtype D reacted with all 4 of the peptides. An additional five of the subtype D sera reacted with the MN peptide and the two Ugandan subtype A and subtype D consensus peptides. Six sera (including 3013 subtype C) reacted with 2 of the peptides. Cross-subtype antibody binding was seen in 22 of the 23 samples in this group of antisera showing that there was no subtype specific binding.

TABLE 2. BINDING OF ANTIBODIES TO V3 LOOP SPECIFIC PEPTIDES.

ANTISERUM/ PLASMA (Subtype)	PEPTIDES USED IN ASSAYS			
	MN	UG A	UG D	U31
C6080 (A)	0.56	1.29	1.57	0.3
UG92001 (D)	0.76	1.12	1.88	-
W15 (D)	1.09	1.03	2.19	-
C972 (D)	1.12	0.87	1.94	-
C971 (D)	1.42	0.41	0.81	-
U1685 (D)	-	1.04	2.29	0.87
UG92031 (D)	0.96	-	-	-
UG92035 (D)	-	-	-	-
3004 (D)	1.12	0.99	1.44	0.68
3011 (A)	1.08	1.5	1.63	-
3012 (A)	-	0.57	0.99	-
3013 (C)	-	0.47	0.75	-
3018 (D)	0.31	-	0.78	-
3025 (D)	0.89	1.3	1.8	0.9
3028 (A/D)*	1.65	1.09	1.39	-
3029 (D)	1.86	-	1.44	-
3032 (D)	0.58	0.93	1.3	0.81
3046 (D)	0.74	0.72	0.58	0.44
3048 (D)	0.46	-	1.33	-
3049 (D)	1.1	0.53	0.58	-
3050 (D)	0.94	0.4	0.69	0.5
3052 (A)	-	0.36	0.6	-
3053 (A)	0.97	0.86	0.73	-

Absorbancy readings (8=450nm) were taken at 1:200 dilutions of plasma.
* (*gag/env*).

Sequential serum samples spanning a 2 year period were obtained from the MRC Repository at UVRI. The peptide binding activities of these sera, from 4 members of the Natural History cohort in rural SW Uganda infected with subtype D viruses, were assayed using the whole V3 loop peptides and the results are shown in Table 3. The 3004 sera reacted with all 4 of the peptides used in the assay; however, the peptide binding activities with all 4 peptides decreased in the 2 year period between the first and last sample: the ELISA readings with the UG D peptide fell by about 50% over this period, much less than readings obtained with the other peptides, which had fallen by between 74% and 87%. The 3025 sera also reacted with all 4 peptides, though they reacted more strongly with the Ugandan consensus peptides than with the MN peptide. A decrease in peptide binding activities was also seen in later samples of this series of sera, but the reduction of binding to the MN and UG D peptides was less than that seen with the UG A and U31 peptides. The reactivity to the U31 peptide was lost completely over the 2 year period. The 3029 sera reacted with the MN and UG D peptides; over the 2 year sampling period the reactivity to the MN peptide decreased by half although that with the UG D peptide remained constant. The 3048 sera reacted with the UG D peptide and very weakly with the MN peptide. The reactivity with the UG D peptide remained constant over the 2 year period, but the MN reactivity was lost after 1 year. These results showed that the peptide binding activity of serum samples from an individual may vary with time: these variations were gradual decreases in peptide binding activity with one or more of the peptides.

TABLE 3. BINDING OF ANTIBODIES TO V3 LOOP SPECIFIC PEPTIDES.

PLASMA /DATE (Subtype)	PEPTIDES USED IN ASSAYS			
	MN	UG A	UG D	U31
3004/18.11.93 (D)	1.1	0.87	1.27	0.56
3004/03.11.94 (D)	0.33	0.23	0.93	0.3
3004/23.10.95 (D)	0.15	0.12	0.67	0.15
3025/22.05.94 (D)	0.48	1.04	1.26	0.9
3025/05.07.95 (D)	0.11	0.18	0.83	0.11
3025/09.04.96 (D)	0.31	0.25	0.95	-
3029/22.02.94 (D)	1.3	-	0.86	-
3029/05.09.95 (D)	0.4	-	0.84	-
3029/21.02.96 (D)	0.32	-	0.78	-
3029/06.06.96 (D)	0.61	-	0.77	-
3048/26.05.94 (D)	0.34	-	0.74	-
3048/13.06.95 (D)	-	-	0.67	-
3048/04.96 (D)	-	-	0.69	-

Absorbancy readings (8=450nm) were taken at 1:200 dilutions of plasma.

Peptides:

MN:
CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC

UG A:
CTRPNNNTRKSVHIGPGQTFYATGEIIGDIRQAHC

UG D:
CTRPYNNTRQSTHIGPGQALYTTNIIGDIRQAHC

U31:
CTRPYYNIRQRTPIGLGQALYTTKGRGTTKVIGQAHC

As the majority of antisera were cross reactive with V3 loop peptides based on subtype A and D and MN sequences it seems unlikely that the subtype of the infecting virus could be distinguished by relatively simple peptide-based assays. It was obviously a serious problem that a very high proportion of the sera reacted strongly with the MN peptide, and also with peptides encoded by some other subtype B viruses (data not shown). To determine whether the peptide based assays could be made more specific, the phenomenon of cross-reactive antisera was investigated in greater detail.

3.2 Investigation of Antiserum Cross-reactivity

The following experiments were done to determine whether the cross-reactivities towards the V3 loop peptides were due to the presence of antibody molecules with very broad specificities or to a mixture of antibodies with more restricted specificities.

3.2.1 Competitive Inhibition Assays

Sera were pre-incubated with peptides before reaction with peptide-coated assay plates. The results for serum U5055, which reacted with all the peptides used in this study, are shown in figure 10. Its reactivity towards the MN peptide (Fig.10a/) was almost completely inhibited by pre-incubation with MN peptide at concentrations as low as 160ng/ml and MN-peptide reactivity was also inhibited by the UG D peptide which gave 50% inhibition at a concentration of 1.25μg/ml. High concentrations (>10μg/ml) of the UG A and U31 peptides gave little or no inhibition of antibody binding to the MN peptide.

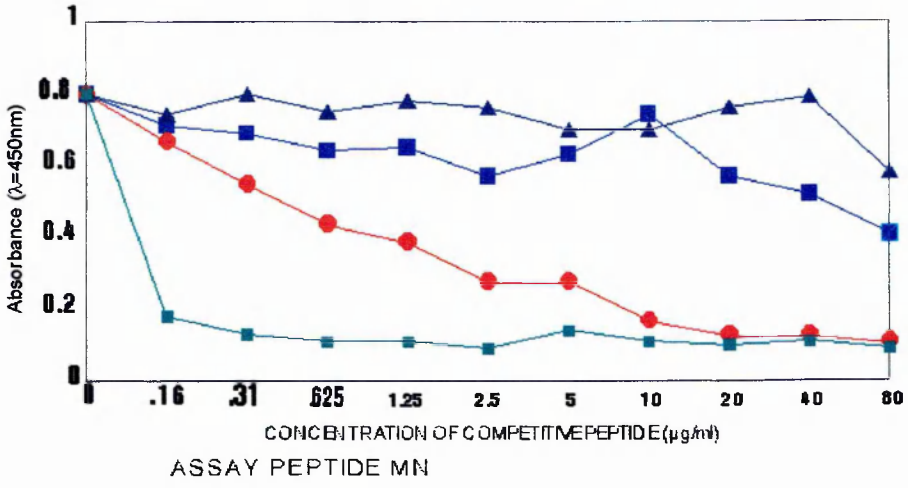
Similarly, the reactivity of serum U5055 towards the UG D peptide (Fig.10b/) was strongly inhibited by pre-incubation with low concentrations (160ng) of the autologous peptide, but high concentrations (5-10μg/ml) of the MN and UG A peptides were required to give 50% inhibition and the U31 peptide did not inhibit.

Figure 10. Competitive inhibition assays with antiserum U5055 (subtype D) at 1:500 dilution. Serum reactivities with peptides MN, UG D and U31 after pre-incubation with peptides MN, UG A, UG D and U31. Each graph shows the absorbance (λ=450nm) obtained in the standard antibody binding assay plotted against the concentration of the competitive peptides.

Figure 10

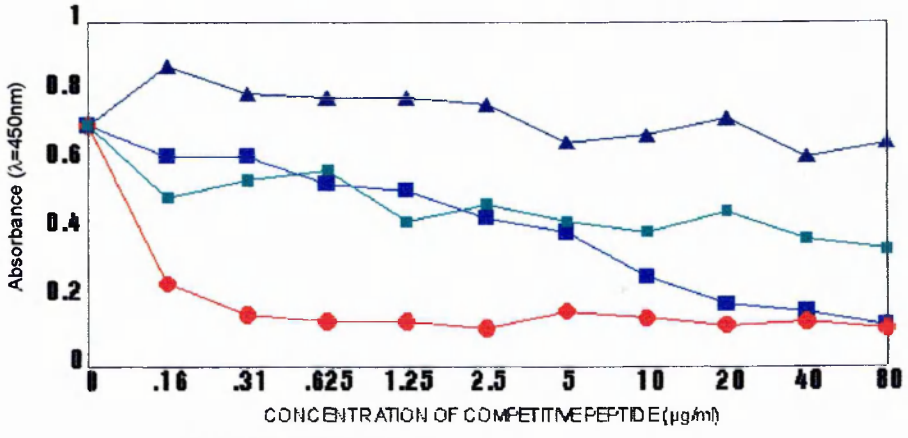
SERUM U5055 1:500

a/



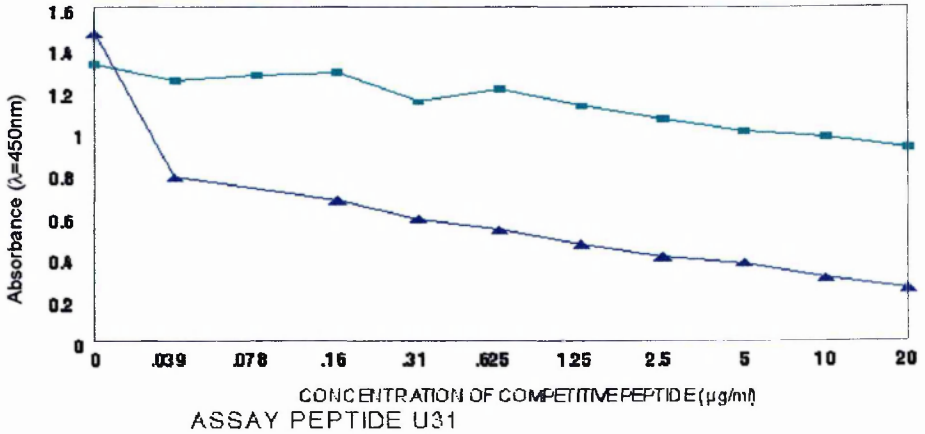
ASSAY PEPTIDE MN

b/



ASSAY PEPTIDE UG D

c/



ASSAY PEPTIDE U31

COMPETITIVE PEPTIDES

MN U31 UG D UG A

Furthermore, reactivity of serum U5055 towards the U31 peptide was inhibited by pre-incubation with the autologous peptide 50% inhibition at 160ng, but not by the MN peptide (20% inhibition at 5μg/ml) as shown in Fig.10c/.

Competitive inhibition of antibody binding was also examined for antiserum ACP 37026, the results are shown in figure 11.

Antibody binding to the MN peptide (Fig.11a) was completely inhibited by pre-incubation of the serum with three of the peptides; UG A inhibited at 39ng/ml, MN at 160ng/ml and UG D at 10μg/ml. Pre-incubation with the U31 peptide did not inhibit binding to the MN peptide.

Antibody binding to the UG A peptide (Fig.11b) was completely inhibited by pre-incubation with 78ng/ml of UG A peptide or with 20μg/ml of UG D: pre-incubation with the MN peptide inhibited binding of the UG A peptide by 75% at 20μg/ml, but pre-incubation with the U31 peptide did not inhibit binding to the UG A peptide.

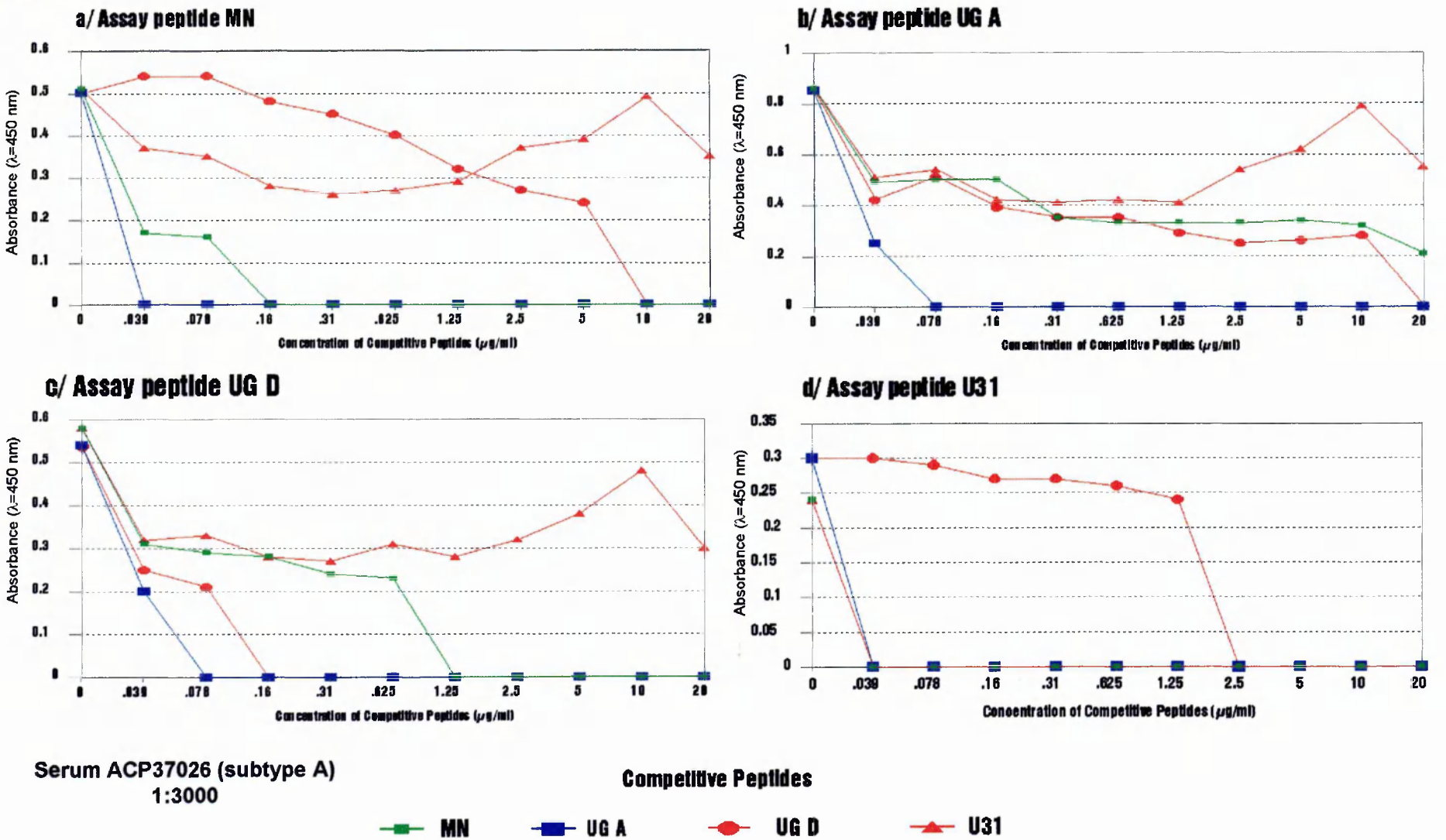
Antibody binding to the UG D peptide (Fig.11c) was completely inhibited by pre-incubation with 78ng/ml of the UG A peptide, with 160ng/ml of the UG D peptide or with 1.25μg/ml of the MN peptide; again the U31 peptide did not inhibit binding.

Antibody binding to the U31 peptide (Fig.11d) was completely inhibited by all four of the assay peptides. 100% inhibition was obtained after pre-incubation with the U31, MN and UG A peptides at 39ng/ml and by the UG D peptide at 2.5μg/ml.

These data indicate that the serum U5055 contained antibodies with relatively restricted specificities and serum ACP 37026 contained more broadly reactive antibodies. Serum U5055 contained two distinct groups of antibodies, those which bound the MN and UG D peptides and another group which bound to the U31 peptide. The peptide inhibition studies suggested that serum ACP 37026 also contained two groups of antibodies. One group with a broad specificity had a high affinity for the UG A peptide, with a slightly lower affinity

Figure 11. Competitive inhibition assays with antiserum ACP 37026 (subtype A) at 1:3000 dilution. Serum reactivities with peptides MN, UG A, UG D and U31 after pre-incubation with peptides MN, UG A, UG D and U31. Each graph shows the absorbance (λ=450nm) obtained in the standard antibody binding assay plotted against the concentration of the competitive peptides.

Figure 11


a/ Assay peptide MN
Absorbance (λ=450 nm)
Concentration of Competitive Peptides (μg/ml)
b/ Assay peptide UG A
Absorbance (λ=450 nm)
Concentration of Competitive Peptides (μg/ml)
c/ Assay peptide UG D
Absorbance (λ=450 nm)
Concentration of Competitive Peptides (μg/ml)
d/ Assay peptide U31
Absorbance (λ=450 nm)
Concentration of Competitive Peptides (μg/ml)
0 .039 .078 .16 .31 .625 1.25 2.5 5 10 20
Serum ACP37026 (subtype A)
1:3000
Competitive Peptides
MN
UG A
UG D
U31

for the MN peptide and a low affinity for the UG D peptide: these antibodies were not inhibited by pre-incubation with the U31 peptide. A second, U31-reactive group of antibodies in serum ACP 37026 also appeared to be broadly reactive, binding strongly to MN and UG A peptides and to a lesser degree to the UG D peptide. This suggests that the U31 antibodies in this serum had lower specificities than the U31-reactive antibodies in serum U5055.

A further difference between the two sera, shown by these competitive inhibition assays, was that the ACP 37026 serum had a higher affinity than the U5055 serum for the UG A peptide, even though the two sera gave similar titres with this peptide in the standard assay (Table 1). The difference in affinities may not be especially surprising as the ACP 37026 serum was obtained from a person infected with a subtype A virus and the U5055 serum came from an AIDS patient infected with a subtype D virus.

As some of the data obtained in these experiments was difficult to interpret, an antibody adsorption method was devised to physically separate antibodies with different V3 loop peptide binding specificities. This method was used to obtain further evidence for the presence of restricted antibody specificities within an antiserum. Unfortunately due to the lack of individual antiserum volumes available from Uganda I used different antisera in these experiments. The peptides used in these adsorption assays were chosen because the antisera had good reactivities to them in the antibody binding assay. With hindsight it might have been tidier to have been able to use the same antisera with the same peptides for the competitive and adsorption assays.

3.2.2 Adsorption of Antibodies to Insolubilized Peptides

Antibodies were adsorbed by filtering antisera through columns of Sepharose coupled with V3 loop peptides (the 16-19mers spanning the loop apex, as shown in Section 2.2) encoded by MN, Z3, U31 or U1685 proviruses: a column containing no peptide served as a control. The residual activities in the filtrate were then titrated in the standard assay. The results obtained with serum U462 (subtype D) show that the MN-reactive antibodies were adsorbed by the MN-

peptide column (Fig.12.i.a) and the Z3-reactive antibodies were adsorbed by the MN- and Z3-peptide columns (Fig.12.i.b).

The U31 peptide column adsorbed the U31-reactive antibodies from serum U462 (Fig.12.i.c) and both the U31- and U1685-peptide columns removed these antibodies from serum U1685 (subtype D) (Fig.12.i.d). Similarly, the U1685-reactive antibodies were removed from this serum by adsorption with either the U31- or U1685-peptide columns (Fig.12.ii.a).

However, the U31-reactive antibodies in these two sera were not adsorbed by the MN-peptide column (Fig.12.i.c & d), nor by the Z3-peptide column (Fig.12.i.c). The results obtained with serum P724 (unknown subtype) show that MN-reactive antibodies were adsorbed by the MN-peptide column and, in contrast to the results obtained with serum U462, also by the Z3-peptide column (Fig.12.ii.b). Similarly, the Z3-reactive antibodies were adsorbed by the MN- and Z3-peptide columns (Fig.12.ii.c).

The results obtained from the competitive inhibition assays and the antibody adsorption experiments indicate that some antisera contain antibodies with restricted specificities and that the specificities can vary between different antisera. For example, the MN-reactive antibodies in serum U462 were not adsorbed by the Z3-peptide column which, however, adsorbed these antibodies from serum P724. So the MN-reactive antibodies in these two sera had rather different specificities.

Figure 12i and 12ii. Reactivities of antisera U462 (subtype D), U1685 (subtype D) and P724 (unknown subtype) after adsorption to insolubilized peptides MN, Z3, U31 and U1685 coupled to Sepharose columns: the control columns contained non-derivitized Sepharose and are shown on each graph with a black filled square marker. Each graph shows the absorbance (λ=450nm) against the reciprocal of the antiserum dilution for the assay peptide used (MN, Z3, U31 or U1685) in the antibody binding assay.

Figure 12i

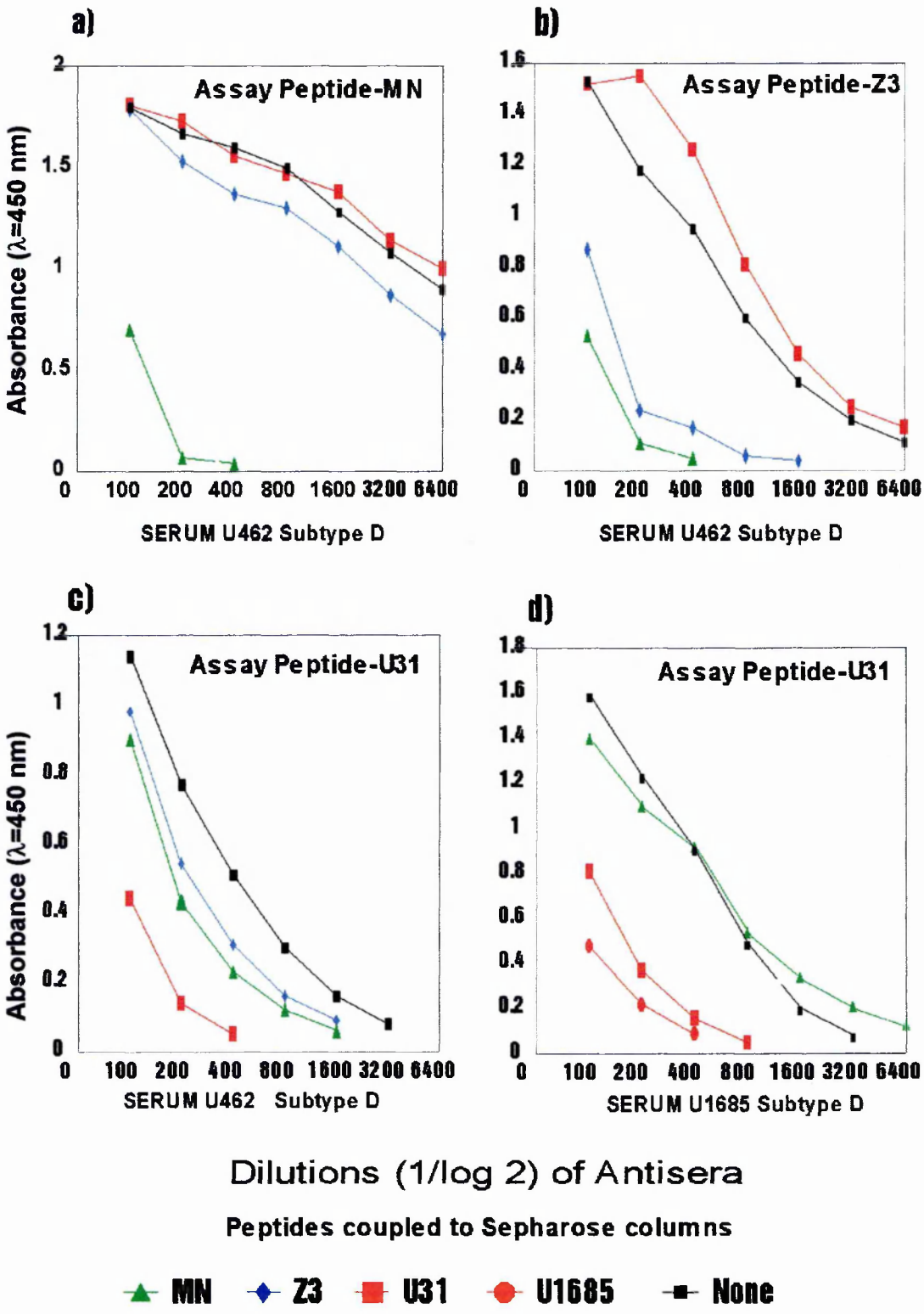
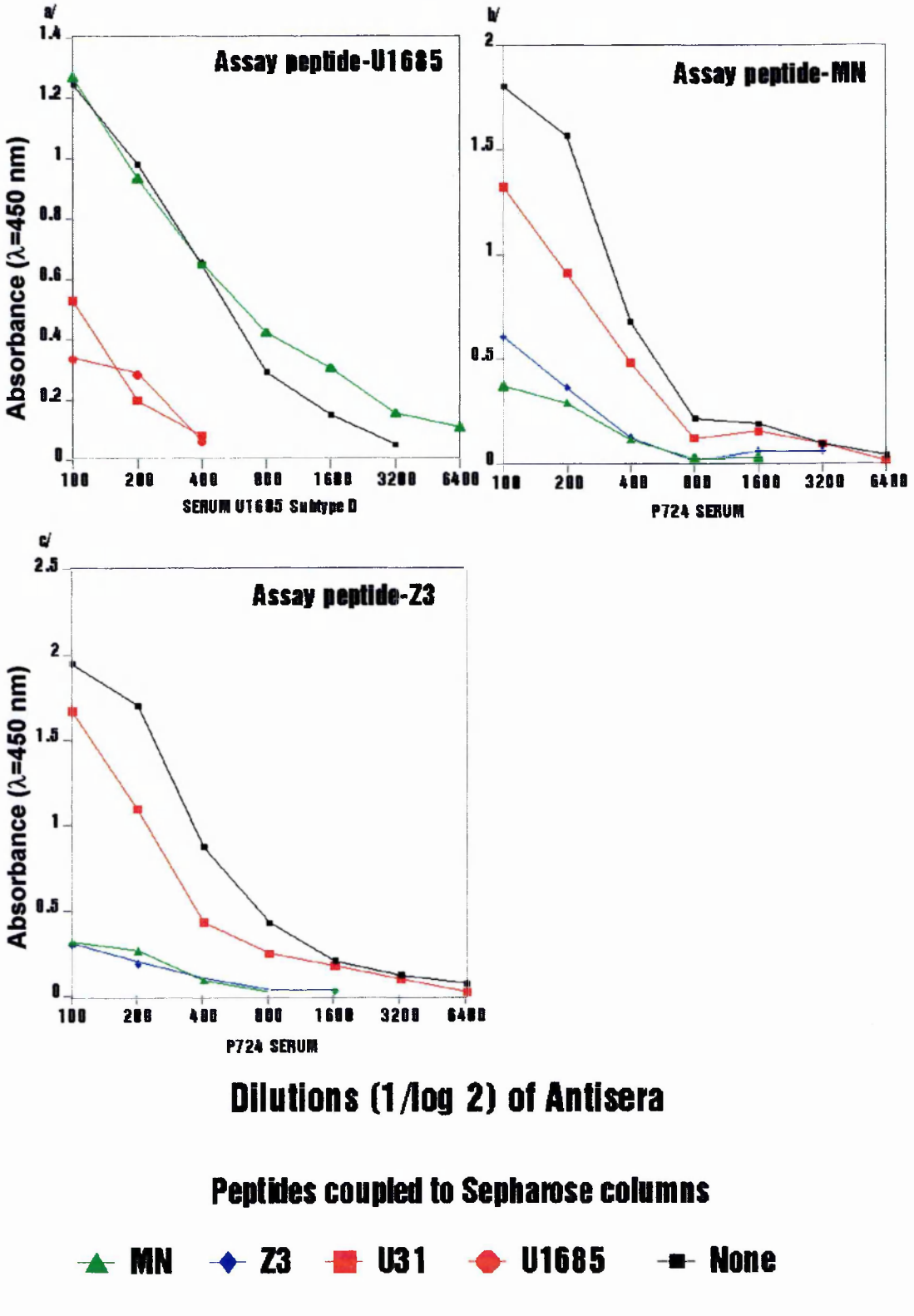

a)
Assay Peptide-MN
Absorbance (λ=450 nm)
SERUM U462 Subtype D
b)
Assay Peptide-Z3
SERUM U462 Subtype D
c)
Assay Peptide-U31
Absorbance (λ=450 nm)
SERUM U462 Subtype D
d)
Assay Peptide-U31
SERUM U1685 Subtype D
Dilutions (1/log 2) of Antisera
Peptides coupled to Sepharose columns
MN
Z3
U31
U1685
None

Figure 12ii


a/
Assay peptide-U1685
Absorbance (λ=450 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
100
200
400
800
1600
3200
6400
SERUM U1685 Subtype D
b/
Assay peptide-MN
2
1.5
1
0.5
0
P724 SERUM
c/
Assay peptide-Z3
2.5
P724 SERUM
Dilutions (1/log 2) of Antisera
Peptides coupled to Sepharose columns
MN
Z3
U31
U1685
None

3.2.3 Determination of the Frequency of V3 loop Binding Antibodies

The data from the competitive inhibition assays and the antibody adsorption experiments showed that many Ugandan sera contained antibodies reactive against at least 2 variants of the V3 loop apical epitopes, e.g. the MN/UG D and U31 epitopes. To determine the frequency of antibodies reactive with V3 loop peptides encoded by MN, U31, and the UG A and UG D consensus sequences sera were obtained from 107 Ugandan AIDS patients and 123 sero-positive asymptomatic persons (as detailed in the Materials and Methods Section 2.1). These sera were assayed and the antibody specificities are summarized in Table 4.

Most (79%) of the sera from the AIDS patients reacted with the MN peptide, 57% reacted with the UG A apex peptide, 57% with the UG D apex peptide and 22% with the U31 peptide. Seventeen of the sera did not react with any of the peptides, 19 with only one peptide, 23 with 2, 29 with 3 and 19 with all 4 peptides. All but one of the singly-reactive sera and all except 4 of the doubly-reactive sera reacted with the MN peptide. Although equal numbers of sera reacted with both of the Ugandan consensus peptides, several sera (14%) reacted with only one of them.

A similar pattern was given by sera from the asymptomatic persons, although the proportion reacting with the MN or U31 peptides (67% and 14%, respectively) was slightly lower than for sera from AIDS patients (79% and 22%, respectively): the difference was statistically significant ($p<0.05$) for the MN but not the U31 peptide. Although more sera in this group were singly-reactive - 32 (25%) compared with 19 (17%) of the sera from the AIDS patients, this difference was not statistically significant ($p=0.067$). However, many of the singly reactive asymptomatic sera reacted with either the UG A or UG D consensus peptides (4.9% and 10.5%, respectively), compared with 1% and 0%, respectively for sera

TABLE 4. Comparison of Peptide Reactivities of Ugandan Sera from 1985/6 and 1990/2 and from AIDS and Asymptomatic Persons.

SERA (number in group)	Peptides			
	MN	UG A	UG D	U31
1985/86 (96)	73 (76%)	67 (70%)	61 (64%)	22 (23%)
1990/92 (117)	79 (68%)	57 (49%)	64 (55%)	16 (14%)
AIDS Patients (107)	85 (79%)	61 (57%)	61 (57%)	24 (22%)
Asymptomatic Persons (123)	83 (67%)	75 (61%)	75 (61%)	17 (14%)
All HIV-1^+ Sera in Study (230)	168 (71%)	136 (59%)	136 (59%)	41 (19%)

Figures expressed as numbers (and percentage) of sera reacting with V3 loop peptides.
N.B. 17 of the AIDS patients / Asymptomatic persons were not included in the 1985/86 and 1990/92 sample groups.

from the AIDS patients: these differences were statistically significant ($p<0.05$) and are summarized in figure 13. In a sub-group of 54 asymptomatic mothers 18 (33.3%) of the sera were singly-reactive: 4 (7.4%) reacted with MN, 12 (22.2%) with UG A and 2 (3.7%) with the UG D consensus peptides. This data indicates that in many Ugandans the early antibody responses to infection were towards either the UG D or MN types of epitope.

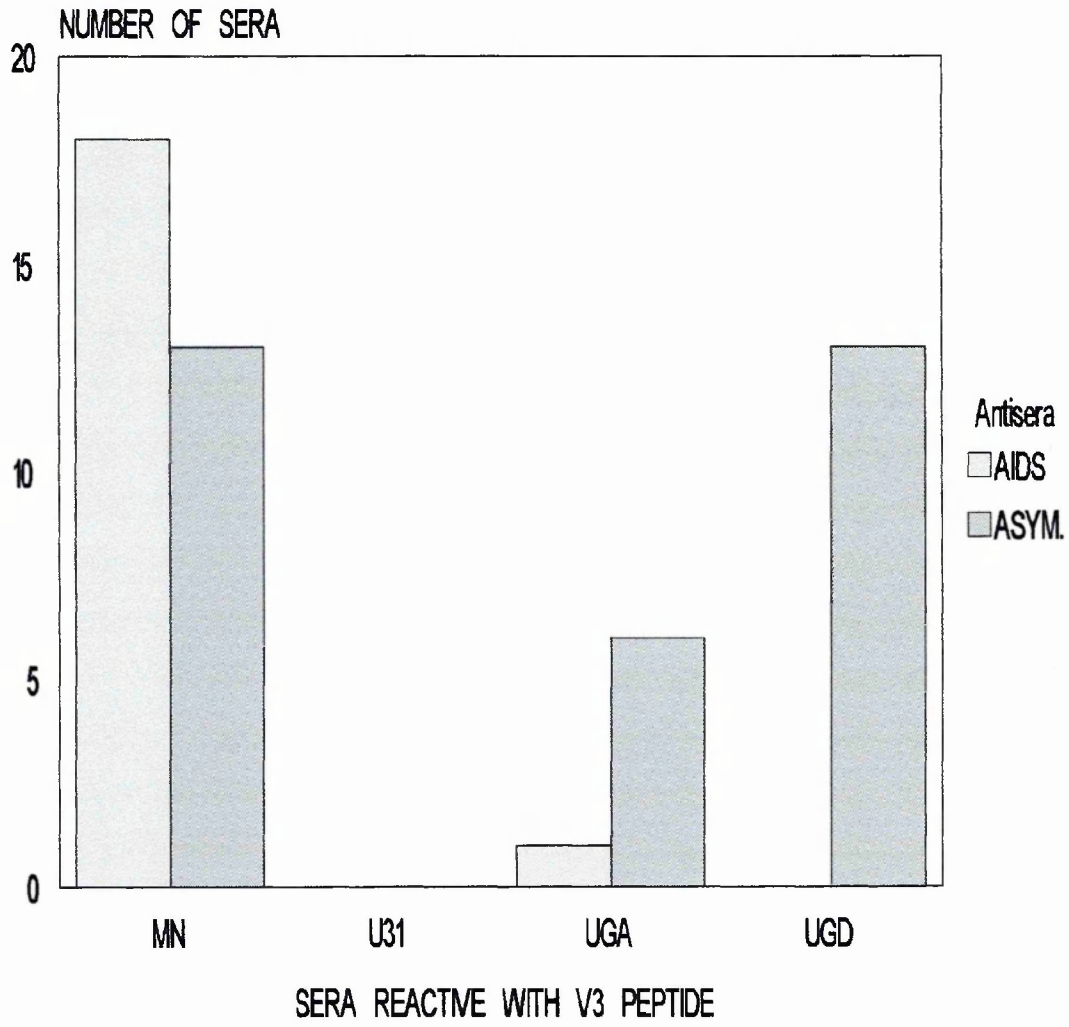
A key question concerning the HIV epidemic is whether the ratio of the different viral subtypes is fairly constant or whether it is changing due to the more rapid spread of one or more subtypes of virus. To address this question, I compared the peptide-binding specificities of sera taken in 1985/6 with those taken in 1990/2: the data is summarized in Table 4. Although the two groups of sera showed similar overall reactivities with the MN and UG D consensus peptides, 70% of the 1985/6 sera but only 49% of the 1990/2 sera reacted with the UG A consensus peptide; this difference was significant ($p<0.005$). As shown in Table 4, about a quarter of the sera sampled in 1985/6 reacted with the U31 type peptide, many with high titres (Table 1): a lower proportion of the 1990/2 sera reacted with this peptide but the number of samples was too low for the difference to be statistically significant.

Some of the viruses isolated from the 1985/6 patients had V3 loop sequences similar to HIV-1_{U31} (Oram *et al.*, 1991) which is not typical of the more recent Ugandan proviruses (Albert *et al.*, 1992; Bruce *et al.*, 1994). Surprisingly, 20% of the 1990/2 sera, compared with only 7% of the 1985/6 sera, did not react with any of the peptides; the difference was statistically significant ($p<0.05$).

The data obtained using human sera revealed a complex pattern of serological responses towards the V3 loop region of the glycoprotein. Comparison of the results obtained with sera from AIDS cases with those from asymptomatic persons (Fig.13) indicated that antibody specificities broadened with time, making it difficult to develop a subtype specific assay.

Figure 13. This bar chart illustrates the number of AIDS and asymptomatic antisera singly-reactive by the antibody binding assay to the V3 loop peptides MN, UG A, UG D or U31.

Figure 13


NUMBER OF SERA
20
15
10
5
0
MN
U31
UGA
UGD
SERA REACTIVE WITH V3 PEPTIDE
Antisera
AIDS
ASYM.

The following experiments were performed to determine whether human sera recognized different regions of the whole V3 loop peptides.

3.2.4 Assays Using Overlapping Peptides Encoded by Parts of the V3 loop

To investigate the possibility that there may be more than one antigenic epitope within the V3 loop, antibody binding assays were carried out using overlapping peptides containing parts of the V3 loop. For these experiments antisera were selected which cross-reacted with the V3 loop peptides MN and UG D irrespective of the genetic subtype of the autologous virus. These antisera were assayed with an eight amino acid peptide corresponding to the MN apex and with three overlapping peptides spanning the UG D V3 loop sequence: Partial V3 loop peptides were also synthesized for the terminal regions of the MN V3 loop. However, as nuclear magnetic resonance revealed that the terminal region partial V3 loop MN peptides contained a high level of undesirable peptide sequences, these MN terminal region V3 loop peptides were unsuitable for use. Further attempts to synthesize these peptides at CAMR were unsuccessful. The peptides used and the results of these assays are shown in Table 5. Of the 13 antisera assayed 12 reacted with each of the two whole V3 loop peptides, only 4 reacted with the MN apex peptide, 2 reacted with the UG D apex peptide, 4 with the UG D amino terminal peptide and 11 reacted with the UG D carboxy terminal peptide. The reactivity of many sera with the carboxy terminal peptide indicates that it contains the main antigenic epitope of the V3 loop. The reactions with the apex peptides and the amino terminal peptide indicate that other epitopes are also present in this region.

Hence, from the data shown in Tables 1, 2 and 5 it appeared that the length of a peptide, i.e., the number of residues, also had an important effect on the specificity of antibody binding. Thus, whereas many of the sera tested with the 16-17mer V3 loop apex peptides reacted much less strongly with the UG A peptide than with the MN peptide (Table 1) many reacted equally strongly with both of the corresponding whole V3 loop peptides (Table 2). One serum, U1685, was tested with both the 17/16-mer loop apex (Table 1) and whole loop peptides (Table 5), which gave quite different results. The serum reacted very

TABLE 5. BINDING OF ANTIBODIES TO PARTIAL V3 LOOP PEPTIDES.

PEPTIDES SERA (Subtype)	MN V3 LOOP	MN APEX	UG A V3 LOOP	UG D V3 LOOP	UG D APEX	UG D NH_3 TERM	UG D COOH TERM
U3529	1.48	-	1.57	1.82	-	-	2.36
C6080 (A)	0.56	-	1.29	1.57	-	-	0.43
UG92001(D)	0.76	-	1.12	1.88	-	-	-
W15 (D)	1.09	-	1.03	2.19	-	-	0.37
C972 (D)	1.12	0.77	0.87	1.94	0.57	0.57	0.62
C971 (D)	1.42	0.58	0.41	0.81	-	-	0.58
U4133 (D)	0.72	-	-	-	-	0.98	1.98
U4132 (D)	0.9	-	-	1.07	-	1.27	2.13
U455 (A)	0.95	-	0.7	1.5	0.39	-	0.69
U1665 (D)	1.12	0.66	1.02	2.09	-	-	1.37
U1685 (D)	-	-	1.04	2.29	-	0.56	1.42
U393	1.63	0.54	1.24	1.93	-	-	-
U383	0.94	-	1.01	1.94	-	-	0.53

Absorbancy readings (8=450nm) were taken at 1:200 dilutions of sera.

Peptides:

MN:

CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC

MN APEX:

HIGPGRAF

UG D:

CTRPYNNTRQSTHIGPGQALYTTNIIGDIRQAHC

UG D NH_3:

CTRPYNNTRQSTHI

UG D APEX:

HIGPGQAL

UG D COOH:

GQALYTTNIIGDIRQAHC

UG A:

CTRPNNNTRKSVHIGPGQTFYATGEIIGDIRQAHC

strongly with the 17-mer MN loop apex peptide and poorly with the 17-mer UG A loop apex peptide peptide (Table 1) but did not react with the whole loop MN peptide although it reacted strongly with the whole loop UG A peptide peptide (Table 5). Further, although this serum bound strongly to the 17-mer MN loop apex peptide (Table 1), it did not bind to the octomeric peptide centred on the MN loop apex (Table 5). Presumably, the majority of the UG A-reactive antibodies bound to an epitope(s) partly or wholly outside the loop apex, and the epitope recognized by the MN-reactive antibodies in the 17-mer loop apex peptide was not fully contained within the octomeric peptide and was inaccessible or had a different conformation in the whole loop peptide. Similar data was obtained with serum U4133 which reacted strongly with the 16-mer UG D consensus loop peptide (Table 1) but did not bind to either the corresponding whole loop or 8-mer apex loop peptide (Table 5). However, this serum reacted with both the amino-terminal and carboxy-terminal UG D peptides (Table 5), indicating that the antibodies recognized epitopes in both of these regions which were possibly not exposed in the whole loop peptide.

The data obtained using human sera and V3 loop peptides of various lengths and sequences revealed a complex pattern of serological responses towards the V3 loop region of the glycoprotein. For example, comparison of the specificities of sera from asymptomatic persons with those from AIDS cases showed that a higher proportion of the former were singly-reactive, indicating that specificities broadened with time. However, somewhat conflicting data was obtained with sequential samples from the Natural History cohort in rural SW Uganda (Table 3), which showed that some antibody titres declined over a 2 year period[3]. Hence, assay results could well depend on the stage of infection and the effect of the time between seroconversion and sampling is unpredictable. These problems, together with those involving the choice of peptides, obviously make it very difficult to develop a subtype specific assay.

[3]Sequential samples were assayed in duplicate, in the same experiment, with the control antisera U3029 and assays were repeated.

3.3 Virus Isolation from Blood Samples

Samples of seropositive blood from persons attending the UVRI clinic in 1990/2 were used to isolate viruses which, after genotypic and phenotypic characterization, could be used in virus neutralization assays using autologous and heterologous plasma. At a slightly later stage (1995) characterized primary isolates and matching sera became available through the MRC-ADP HIV Repository at Cambridge (see above).

3.3.1 Isolation of Viruses from the UVRI clinic

Virus isolation was attempted on 30 samples from the UVRI clinic. The blood samples were separated by Ugandan technicians at UVRI into plasma and leukocyte fractions, as described in Sections 2.8 and 2.9, and frozen at -80°C or in liquid nitrogen, respectively, and then transported to CAMR. Frozen aliquots of the infected leukocytes were thawed and added to PHA stimulated cultures of PBMC and the cultures examined by microscopy for the development of a cpe and by ELISA for the production of p24 antigen. Twelve isolates were obtained: most of the cultures which yielded virus were positive, by either cpe or p24 antigen production, within about 7 days. Two additional primary isolations were made, UG92001 and UG93070, giving a total of 14 isolates from the Entebbe/Kampala area and a 44% rate of isolation. The isolates included 2 subtype A and 7 subtype D viruses and 5 other viruses which were not genotyped as shown in table 6.

Although this isolation rate may appear to be quite low, I required viruses which grew to a high enough concentration of p24 antigen to allow detection after inhibition by antisera in the neutralization experiments (as described in Section 2.9). Therefore, I set the threshold for isolation fairly high at a p24 concentration of 100 pg/ml in the culture supernatant. A few isolates were lost to antibiotic-resistant bacterial infections. These presumably originated from the blood samples, as they occurred in unrelated circumstances. One sample had a viral contamination (confirmed by electron microscopy).

TABLE 6. VIRUS CHARACTERIZATION.

VIRUS ISOLATE (SUBTYPE)	PBMC		MT-2		C8166		JM	H9	U937
	CPE	TITRE	CPE	TITRE	CPE	TITRE	CPE	CPE	CPE
C24 (D)	SI	10^3	SI	10^2	SI	10^2	SI	SI	SI
C971 (D)	NSI	10^3	SI	10^4	SI	10^4	SI	SI	SI
C972 (D)	NSI	10^3	SI	10^3	SI	10^4	SI	SI	SI
C986	SI	10^2	SI	10^2	SI	ND	ND	ND	ND
C6080 (A)	SI	10^2	SI	10^4	SI	10^4	SI	ND	ND
K970	SI	10^1	SI	10^1	SI	ND	ND	ND	ND
K774	SI	10^2	SI	10^2	SI	ND	ND	ND	ND
K359	SI	10^1	SI	10^1	SI	ND	ND	ND	ND
MO47 (A)	NSI	10^3	SI	10^2	SI	ND	SI	ND	ND
UG92001 (D)	SI	10^3	SI	10^2	SI	10^2	SI	SI	SI
UG92031 (D)	SI	10^2	NSI	10^2	NSI	10^2	NSI	NSI	NSI
UG92035 (D)	SI	10^5	SI	10^3	SI	10^5	SI	SI	SI
UG93070	NSI	10^3	NSI	10^2	NSI	10^2	NSI	NSI	NSI
W15 (D)	SI	10^3	SI	10^3	SI	10^5	SI	SI	SI

CPE = Cytopathic Effect ND = Not Done
SI = Syncytium Inducing NSI = Non-Syncytium Inducing

Attempts to isolate some of the viruses directly on PBMC derived from the same infected sample, i.e. without adding feeder PBMC, were not successful, although some cultures showed a short-lived "blip" of p24 antigen. Isolation of virus on autologous cells was not attempted with samples where CD4 cell depletion had been recorded by the clinicians, therefore it was unlikely that lack of CD4 lymphocytes would have contributed to these unsuccessful isolations. The reasons for the absence of virus growth in autologous cells were not investigated but could have been due to the presence of HIV-specific cytolytic T cells in the cultures.

3.3.2 Adaption of Primary Isolates to Cell Lines

The viruses isolated in PBMC cultures were adapted to grow in several T cell lines (MT-2, C8166, JM and H9) and the monocyte line U937 by infecting cultures with supernatants from the primary isolates and monitoring the cultures for virus production, as above. Cell line adapted viruses were obtained from all of the cell lines for each of the primary isolates where adaption was attempted.

The primary isolates, the MT-2 adapted and some of the C8166 adapted viruses were expanded by one or two passages in cultures of PBMC or the appropriate cell line, and the culture supernatants titred, using cultures of PBMC, MT-2 or C8166 cells in microtitre plates, in the virus titration assay described in Section 2.11. The virus isolates obtained and the titres of the expanded culture supernatants are shown in Table 6. This shows that in both PBMC and MT-2 cell cultures most of the viruses grew to relatively low titres (10^2 to 10^3): the lowest titre was 10^1 and the highest 10^5.

3.4 Phenotypic Characterization of HIV-1 Isolates

The primary isolates and cell line adapted viruses were phenotyped by examining infected PBMC or cell line cultures for the development of syncytia. As shown in table 6, 10 of the primary isolates induced syncytia in cultures of PBMC, i.e., they had the SI phenotype, and 4 were non syncytium inducing

(NSI). However, when the corresponding MT-2 adapted viruses were cultured in MT-2 cells, all but two showed the SI phenotype and similar results were obtained with the other cell line adapted viruses. Hence, it appeared that 3 out of 4 of the PBMC isolates which had the NSI phenotype in PBMC cultures must have contained some SI variants which were selected on adaption to growth in the cell lines. Interestingly, one of the viruses which was SI in PBMC cultures was obtained with the NSI phenotype when isolated and tested in the cell lines. These experiments show that some NSI variants can grow quite well in cell lines and in some cases these may even be selected in the presence of SI variants.

The T cell line adapted viruses obtained from the primary isolates had the same phenotype in all of the cell lines tested. Thus, 6 of the variants had the SI phenotype in all the cell lines tested and 2 other variants (UG92031 and UG93070) were both NSI on all the cell lines.

The differences in the phenotypes of PBMC and MT-2 cell grown viruses were also reflected by differences in their susceptibilities to neutralizing antibodies (see below).

3.5 Virus Neutralization Studies

To assess neutralization of isolates of HIV-1 in various cell systems by antisera I decided to measure the reduction in p24 production by the isolates grown with dilutions of antisera against a control (HIV-1 negative) serum. I settled for a dilution range of sera from 1:10 to 1:640 which was chosen to give a pattern of neutralization of the isolates, although in a few of the earlier experiments I used a range from 1:10 to 1:320. I aimed to show with in this range of sera dilutions a maximum neutralization at 1:10 dilution and a much lesser degree of neutralization at 1:640. All of the neutralization assays were carried out using a series of dilutions 1:10 to 1:640 of a HIV-1 negative control serum. When the p24 levels were measured for the assays if there was a greater than 5% variation between the 1:10 and 1:640 dilution readings for the negative control serum and the readings for the virus culture with no added antiserum, then the data were discarded. On all the neutralization plots shown in this thesis the 1:10 and 1:640

negative control serum results are shown. All dilutions were duplicated, and each assay was repeated to verify the results: All the results shown in this thesis were reproducible. Where comparisons were to be drawn directly between sequential virus samples these neutralization assays were always part of the same experiment and were repeated together.

In order to compare the data from these neutralization assays I decided to tabulate the titres at various percentages of neutralization. 50% and 90% neutralization tables revealed less information than 75% neutralization tables. At 50% neutralization too many antisera titres were >640, and many antisera even at 1:10 dilutions did not neutralize the isolates to 90%. Therefore in this thesis I had tabulated my data at 75% neutralization of viral p24 production. Appendix VII illustrates in more detail how I reached this decision. As discussed below the data in Figure 14 and Table 7 illustrate the difficulties in interpretation of neutralization data.

Shallow neutralization curves where the virus was neutralized to around 75% across a range of antiserum dilutions were difficult to interpret. Examples of this are seen below in Figure 15f/ Isolate C971 on PBMCs with sera C972 and U1685, Fig.16i a/ Isolate 3004 with the autologous serum, Fig.16i c/ Isolate 3052 with the serum 3050, Fig.16iii b/ Isolate 3048 with serum 3025, Fig.17 Isolate UG92035 with serum C6080 and Fig.19 Isolate UG92001 with sera C972 and U1685. In these 8 incidences it was difficult to measure a neutralization titre, but in the majority of neutralizations this was not the case. For these 8 neutralizations all the available data for each antiserum was plotted together on one graph (expressed as % p24 produced to allow for inter-test variations) and the neutralization titre was assessed from there. Appendix VIII shows 2 examples of this assessment.

3.5.1 Neutralization of Viruses from the UVRI Clinic

At the time this work was started (late 1992), most laboratories were using cell line adapted viruses in neutralization experiments because it was technically more difficult and time consuming to do experiments with primary isolates in

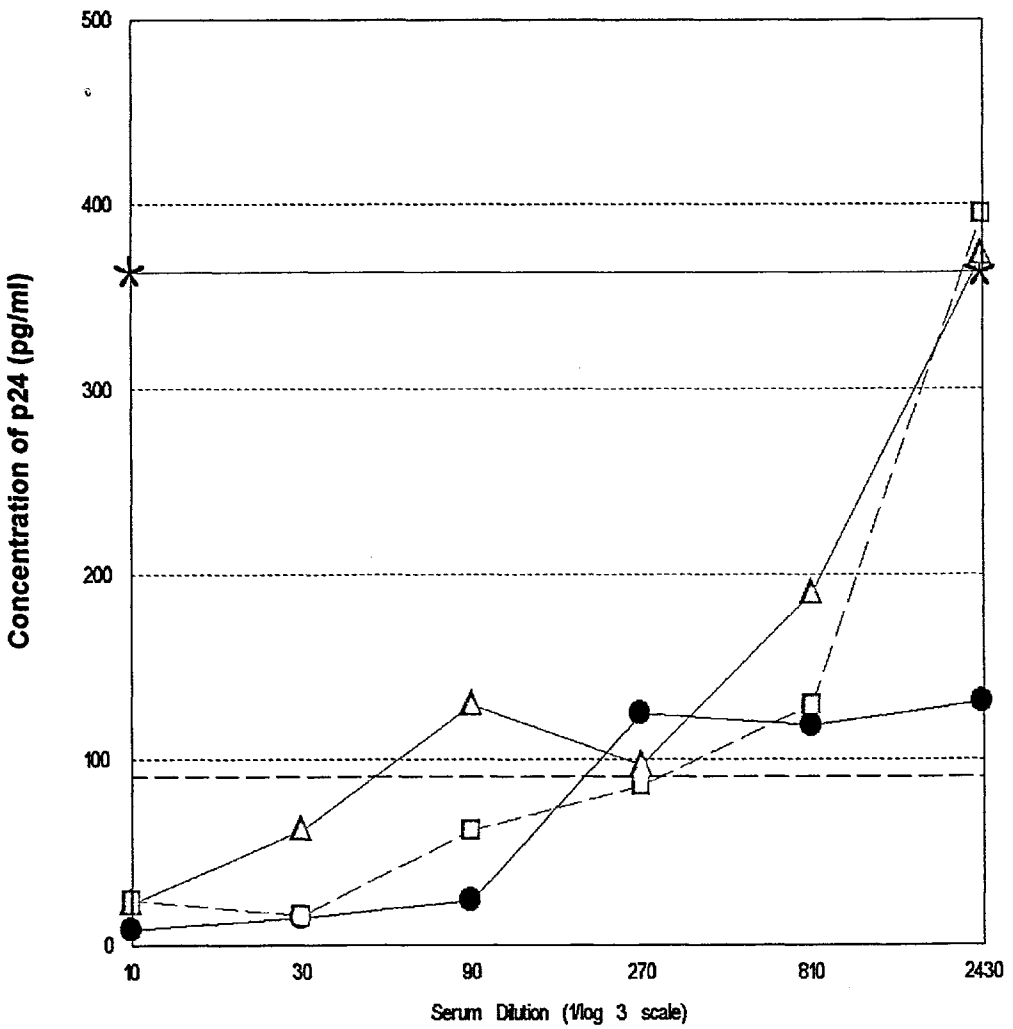
cultures of PBMC (or other primary cells). However, primary isolates were being neutralized in a few laboratories and I was helped by Dr Pontiano Kaleebu, then at St. Mary's Hospital Medical School in London, to set up the assay at CAMR. This enabled me to compare the neutralization data obtained with cell adapted viruses with those obtained with primary isolates from the same blood samples taken in the UVRI clinic.

3.5.1.1 MT-2 cell Adapted Viruses

Neutralization assays in MT-2 cell cultures were carried out with 5 of the Entebbe isolates and HIV-1_{MN} using autologous and heterologous human sera and the HIV-1_{MN} monoclonal antibody. The data obtained in one experiment involving the neutralization of isolate C971 by 3 Ugandan HIV-positive sera and one seronegative control serum is plotted in Figure 14. The neutralization titre was arbitrarily defined as the reciprocal of the dilution that caused 75% reduction of the p24 antigen formation, relative to an HIV-1 negative antiserum control, after 7 days in culture. Figure 14 shows that neutralization by 2 of the 3 sera gave a pattern of correlation between growth inhibition and the (logarithmic) serum dilution whereas the other serum (C6080) gave a marked inhibition of virus growth at much higher dilutions, even though its 75% neutralizing titre was about the same (210 versus 270) as one of the other 2 sera. This type of neutralizing activity, which was not observed in most experiments, is difficult to interpret. This serum gave high neutralizing titres against several viruses (see below) so the effect shown in Fig. 14 could indicate that the antibodies had either a lower specificity or affinity for the C971 virus. It did not appear to be due to a non-specific inhibition of virus growth as it was not observed when serum C6080 was used in some other experiments. The results of the neutralization experiments with MT-2 cell adapted viruses are

Figure 14. Neutralization of HIV-1_{C971} (Subtype D) in MT-2 cell cultures by antisera. The graph shows the concentration of p24 Ag produced in the cultures plotted against the reciprocal of the dilution of the neutralizing HIV-1+ antiserum (subtype shown in brackets). The arbitrarily defined neutralization titre at 75% inhibition of p24 production is indicated by a black dashed line.

Figure 14



- - - = 75% inhibition of p24 production.

summarized in Table 7.a. These data show that 4 out of 5 of the Ugandan sera neutralized the autologous virus, and 5 out of 6 of the Ugandan sera had cross-reactive neutralizing activities with titres greater than 20. Four out of six of the Ugandan sera neutralized the back-adapted HIV-1_{MN} with titres ranging from 40 to 200 and the other 2 sera had weakly neutralizing titres (10-20) towards this virus. Although 4 of the 5 Ugandan viruses tested were neutralized by several Ugandan sera, one (C972) was neutralized by only one (heterologous) serum.
One of the Ugandan isolates HIV-$1_{UG92035}$ and HIV-1_{MN} were neutralized by the monoclonal antibody to the V3 loop apex of HIV-1_{MN} (MNMab). The V3 loop sequences were compared to see if this pattern of neutralization correlated with the amino acid sequences of the neutralized isolates. The V3 loop sequences are shown in Table 7.b. The monoclonal antibody was mapped to a linear epitope on the apex of the V3 loop (HIGPGR) of HIV-1_{MN} (Gorny *et al.*, 1991), shown in bold in Table 7.b. There appeared to be no significant differences in amino acid sequence in this region of the V3 loop between the 2 isolates which were neutralized by the HIV-1_{MN} monoclonal antibody and the 4 isolates which were not neutralized.

3.5.1.2 PBMC Isolates

Neutralization assays were performed using cultures of PBMC with 8 of the Entebbe primary isolates - six SI isolates, two NSI variants, HIV-1_{MO47} and HIV-1_{C971}, and the back-adapted HIV-1_{MN}. The results of these assays are summarized in table 8.
Two of the Ugandan viruses (HIV-1_{C24} and HIV-1_{W15}) and the back-adapted HIV-1_{MN} were fairly strongly neutralized by most of the sera used: the most notable exception being HIV-1_{C24} which was not neutralized by its autologous serum. The susceptibilities of isolates HIV-1_{MO47}, HIV-1_{C971} and HIV-1_{U31} to neutralization varied considerably between different antisera, which gave titres ranging from <10 to >320 for HIV-1_{C971} and HIV-1_{MO47} and between <10 and 160 for isolate HIV-1_{U31}. Isolate HIV-$1_{UG93070}$ was neutralized by 7 antisera, but all with titres #40. Isolate HIV-$1_{UG92001}$ was neutralized by 7 antisera, but 5 of these

TABLE 7.a. NEUTRALIZATION OF UGANDAN ISOLATES IN MT-2 CELL CULTURES.

Sera (Subtype)	Virus Isolate (Subtype)					
	C971 (D)	C972 (D)	C6080 (A)	UG92035 (D)	W15 (D)	MN* (B)
C971(D)	270	-	-	-	20	60
C972 (D)	70	-	10	15	-	80
C6080 (A)	210	-	100	60	200	20
UG92035 (D)	-	-	40	40	40	40
W15 (D)	-	160	10	-	20	10
UG92031 (D)	-	-	40	35	20	120
GB8* (B)	-	-	-	-	-	10
MNMab** (B)	-	-	-	150	-	1000

* Non-Ugandan antiserum/virus isolate. ** Monoclonal antibody to the V3 loop of HIV-1_{MN}. - = Inhibition less than 75%. Figures shown as reciprocal of antiserum dilution giving 75% HIV-1 inhibition as determined by p24 detection.

TABLE 7.b. V3 LOOP SEQUENCES OF HIV-1 ISOLATES IN MT-2 CELL CULTURES.

C971 C T R P Y T N I R Q K T **S I G L G Q** A V Y T T K - R A G Y A G P A Y C

C972 C R R P Y K **N** I R R G T **P I G Q G Q** A I Y T T K A I - G K I G P A H C

C6080 C T R P F N K R R Q T T **P I G L G Q** A L Y T T R Y T T G D I R K A H C

UG92035 C A R P Y Q N K R R R T **S I G Q G Q** A L Y T T R - I T G Y I R Q A H C

W15 C T R P Y H **N** T R Q R T **H I G T G Q** A L Y T T R - I I G D I R K A H C

MN C T R P N Y N K R K R I **H I G P G R** A F Y T T K N I I G T I R Q A H C

TABLE 8. NEUTRALIZATION OF UGANDAN HIV-1 ISOLATES AND HIV-1_{MN} IN PBMC CULTURES.

Sera (Subtype)	Virus Isolate (Subtype)								
	C24 (D) SI	W15 (D) SI	UG92001 (D) SI	MO47 (D) NSI	UG92035 (D) SI	C971 (D) NSI	UG93070 SI	U31 (D) SI	MN* (B) SI
C24 (D)	-	ND	ND	ND	ND	ND	-	ND	ND
W15 (D)	60	>320	20	20	20	20	30	30	>320
UG92001 (D)	160	>320	-	ND	-	40	-	120	>320
UG92031 (D)	>320	>320	20	20	ND	ND	-	ND	ND
UG92035 (D)	80	>320	15	120	ND	>320	-	-	>320
C971 (D)	40	>320	40	10	10	10	30	30	>320
C972 (D)	>320	30	20	20	-	>320	30	40	>320
UG93070	>320	>320	-	ND	ND	ND	40	ND	ND
C6080 (A)	>320	>320	20	120	>320	160	15	80	>320
U1685 (D)	>320	40	40	>320	160	>320	40	160	>320
GB8* (B)	10	>320	-	-	-	-	15	-	>320
MNMab** (B)	-	>320	-	-	-	-	-	-	>320

Figures shown as reciprocal of antiserum dilution giving 75% HIV-1 inhibition as determined by p24 detection.
* = non-Ugandan. ** Monoclonal antibody to the V3 loop of HIV-1_{MN}. ND = Assay not done. - = Inhibition less than 75%.
Shading indicates homologous pairs of viral isolate and antiserum.

had titres of 20, or less. By contrast, HIV-1_{W15} was very sensitive to neutralization, being strongly neutralized by 7 Ugandan antisera and GB8 and MNMab at dilutions of >1:320.

Several Ugandan sera neutralized all or most of the isolates tested, showing high neutralizing titres towards some of them. Thus, serum C972 neutralized 7 out of 8 and sera C971, W15, C6080 and U1685 neutralized all 8 Ugandan viruses tested, plus all 5 sera neutralized the back-adapted HIV-1_{MN}. However, serum UG92001 neutralized only 4 of the 7 Ugandan isolates tested, plus HIV-1_{MN}: like serum C24, it failed to neutralize the autologous virus. As a very small volume of the C24 serum was available, it was not possible to test this serum against a range of viruses.

The 2 subtype B sera, GB8 and the HIV-1_{MN} monoclonal antibody, were very effective against the back-adapted HIV-1_{MN} and the "super-neutralizable" HIV-1_{W15} isolate, but had little or no neutralizing activity against the other Ugandan isolates.

There did not seem to be any genotypic restriction to neutralization between subtype A serum C6080 and the subtype D isolates and the subtype B back-adapted HIV-1_{MN}.

Also, the NSI isolates, HIV-1_{MO47} and HIV-1_{C971}, were as susceptible to neutralization as the SI isolates, showing that there was no significant phenotypic restriction to neutralization.

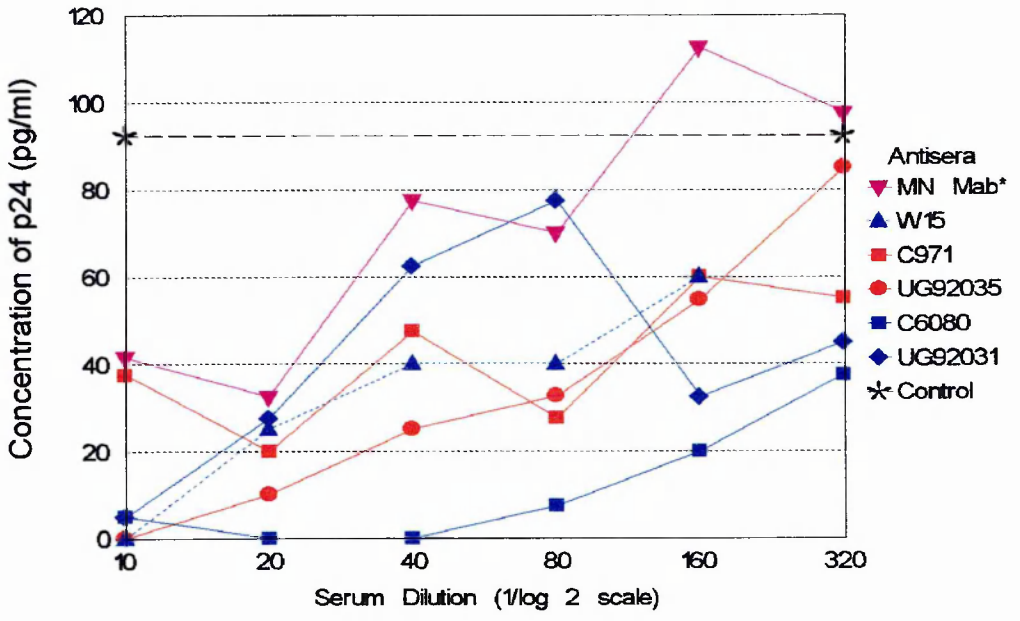
3.5.1.3 Comparison of Neutralization Patterns Shown by MT-2 cell Adapted and Primary Isolates

The neutralization patterns obtained with primary isolates and their MT-2 cell adapted variants are shown in figure 15a/ to f/ and the data from these and additional replication neutralization experiments are summarized in table 9. These data show that there were very marked differences in the neutralization patterns obtained between some primary viruses and the corresponding cell adapted variants. Fig.15a/ and b/ show that the HIV-1_{W15} isolate, like some

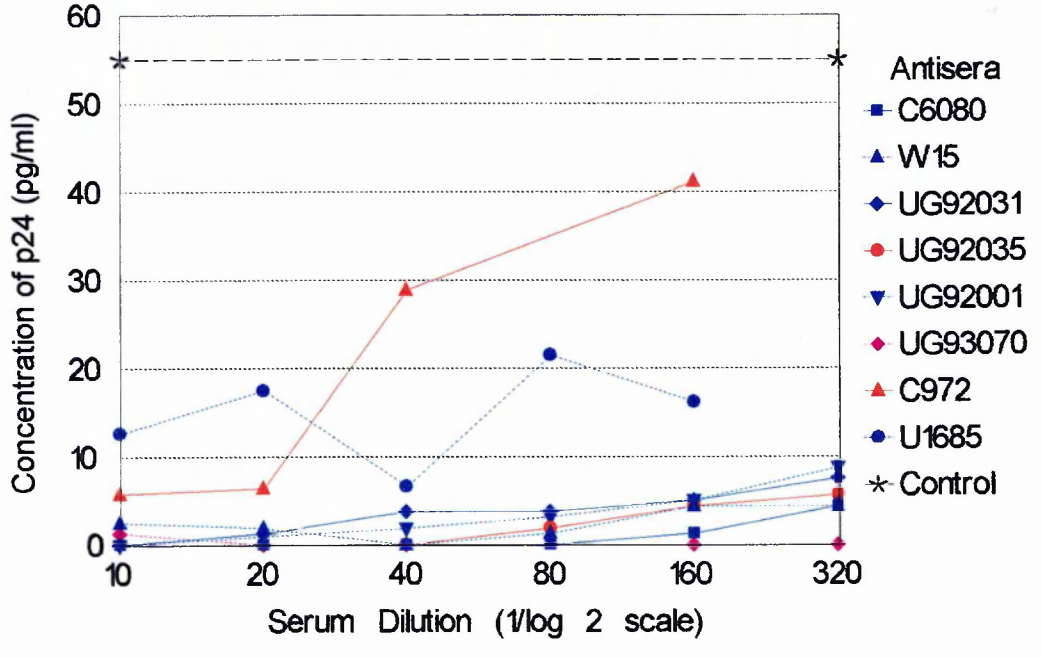
Figure 15. Comparison of the neutralization patterns obtained for primary isolates with MT-2 cell adapted viruses, grown in PBMC and MT-2 cell culture respectively. Fig.15a/ and b/ show HIV-1_{W15} (subtype D), 15c/ and d/ show HIV-1_{MN} (subtype B) and 15e/ and f/ show HIV-1_{C971} (subtype D). The graphs show the concentration of p24 Ag produced in the cultures plotted against the reciprocal of the dilution of the neutralizing antiserum.

Figure 15

a/ W15 virus in MT-2 cell culture



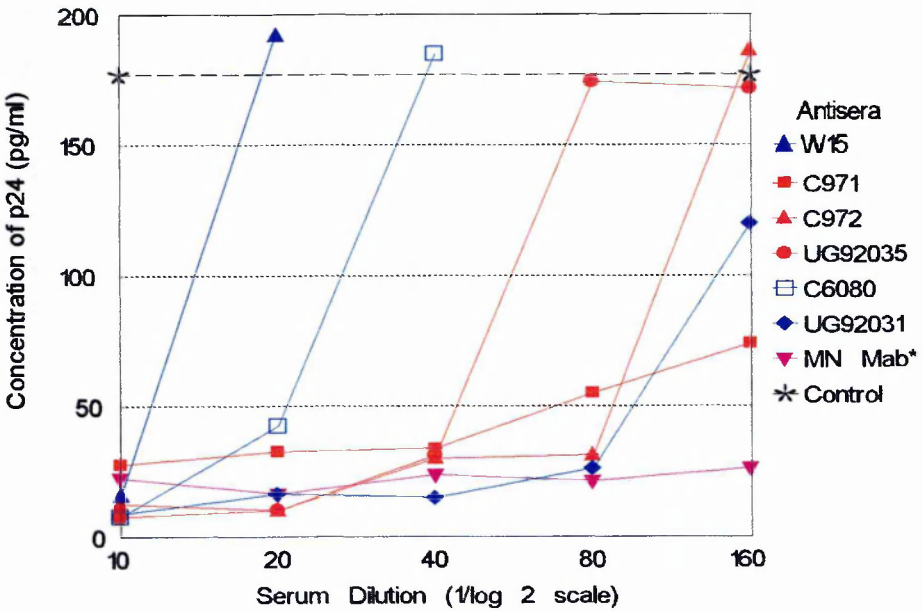
b/ W15 virus in PBMCs



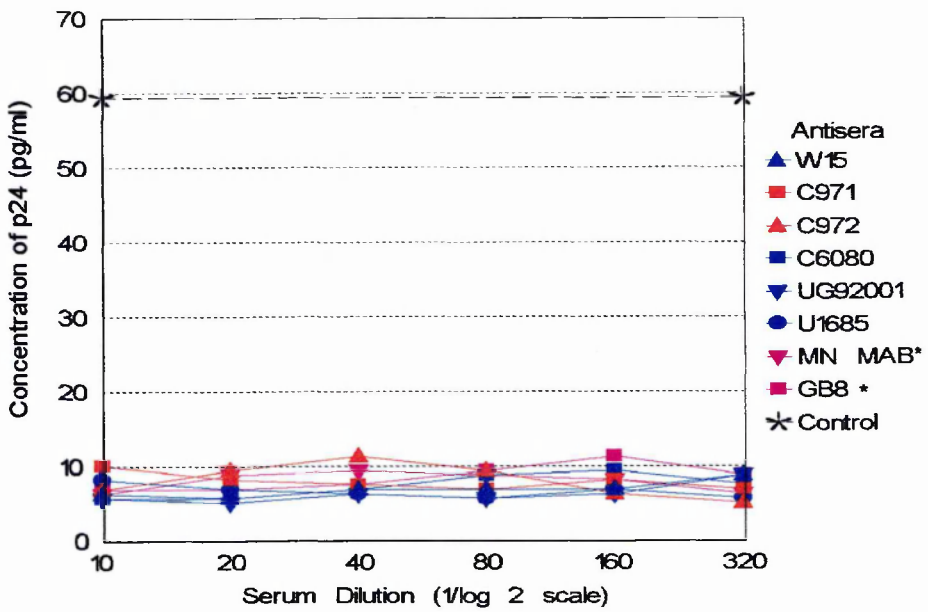
*Non-Ugandan sera

Figure 15

c/ MN virus in MT-2 cell culture



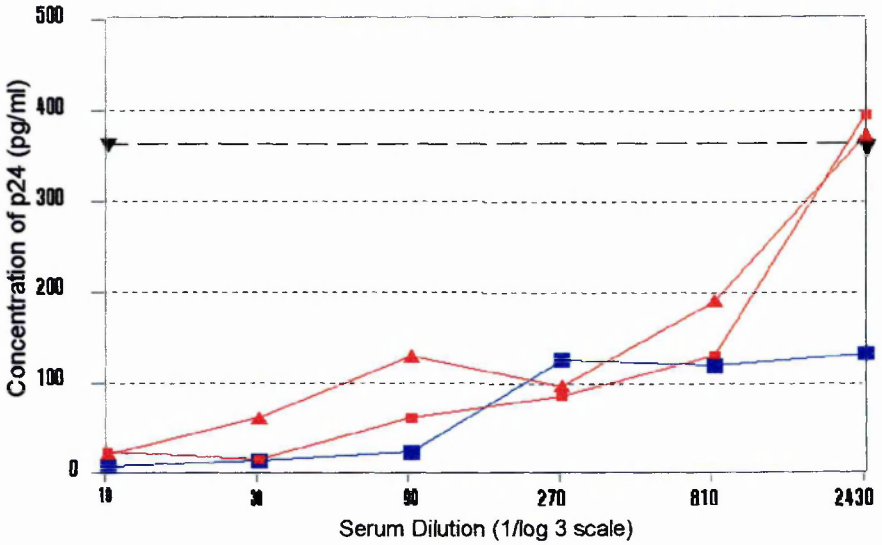
d/ MN virus in PBMCs



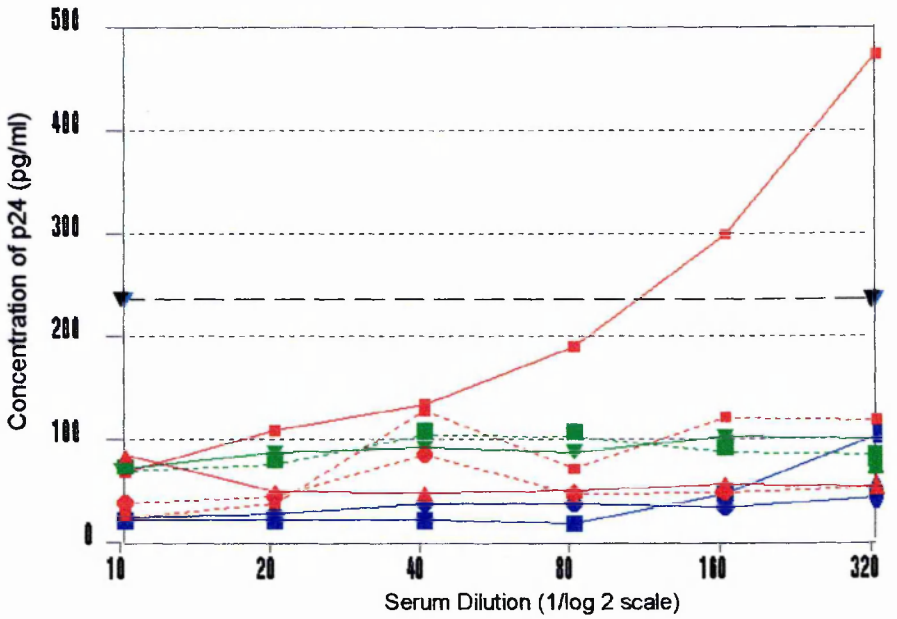
* Non-Ugandan sera

Figure 15

e/ C971 virus in MT-2 cell culture



f/ C971 virus in PBMCs



Antisera

C971 C972 UG92035

MN Mab* W15 C6080

GB8* Control U1685

* non-Ugandan sera

TABLE 9. COMPARISON OF NEUTRALIZATION PATTERNS FOR MT-2 ADAPTED AND PRIMARY ISOLATES OF UGANDAN VIRUSES

Sera (Subtype)	Virus Isolate (Subtype)							
	C971 (D)		W15 (D)		UG92035 (D)		MN* (B)	
	MT-2	PBMC	MT-2	PBMC	MT-2	PBMC	MT-2	PBMC
C971 (D)	270	10	20	>320	-	10	60	>320
C972 (D)	70	>320	-	30	15	-	80	>320
C6080 (A)	210	160	200	>320	60	>320	20	>320
UG92035 (D)	-	>320	40	>320	40	ND	40	>320
W15 (D)	-	20	20	>320	-	20	10	>320
UG92031 (D)	-	ND	20	>320	35	ND	120	ND
GB8* (B)	-	-	-	>320	-	-	10	>320
MN Mab** (B)	-	-	-	>320	150	-	1000	>320

Figures shown as reciprocal of plasma dilution giving 75% HIV-1 inhibition as determined by p24 detection.
ND = Assay not done. - = Inhibition less than 75%.
* Non-Ugandan virus isolate/sera. ** Monoclonal antibody to the V3 loop of HIV-1_{MN}.

other primary viruses, appeared to be much more sensitive than the MT-2 cell adapted variant to neutralization by a wider range of sera. The HIV-1_{MN} variant back-adapted to grow in PBMC was also more susceptible than the MT-2 cell adapted variant (Fig.15c/ and d/). Although the primary and cell adapted variants some of the other viruses, like HIV-1_{C971} (Fig.15e/ and f/) and HIV-$1_{UG92035}$, showed broadly similar neutralization patterns. There were numerous differences in detail: for example, the HIV-1_{C971} primary isolate was sensitive to neutralization by serum UG92035 which did not neutralize its MT-2 cell adapted variant (Table 9). The primary isolate HIV-$1_{UG92035}$ was not neutralized by MNMab, which did neutralize its MT-2 cell adapted variant (Table 9).

Figure 15a/ shows a zig-zag pattern in the p24 concentrations produced by HIV-1_{W15} at increasing dilutions of sera; one possible explanation for this pattern, which was seen in each repeat of this experiment, is that there are a mixture of neutralizing and enhancing antibodies in the antisera (UG92031 and C971). It may have been caused by the action of a component of the growth medium on the specific virus-antibody complexes (Booth, 1977).

All the isolates with high enough titres were used in neutralization assays at a virus input level of 100µl of 100 EPT/ml. I was confident that neutralization titres for antisera could be compared between an isolate neutralized on PBMCs and MT-2 cells, because the titres for most of the isolates were similar in the two cell systems (as shown in Table 6). The greater susceptibility to neutralization of MT-2 grown viruses versus PBMC grown viruses was not predicted by published work. However, it could not have been due to lower infectivity of the isolates on the cell line. Isolates HIV-1_{W15} and HIV-1_{MN} had the same titre in each cell system (see Table 6), so their infectivities would have been the same in the two cell systems. Isolate HIV-1_{C971} had a higher titre in MT-2 cells, therefore it would apparently grow better there if not added in a standard amount of 10 EPTs/ml. Isolate HIV-$1_{UG92035}$ was the only isolate that had a higher titre in PBMCs, therefore it would apparently grow better there if not added in a standard amount of 10 EPTs/ml.

In view of these data, I decided that data obtained with primary isolates cultured in PBMC would be much more relevant to the clinical situation. In the later stages of the study neutralization of Ugandan isolates was continued using low passage primary isolates cultured in PBMC. This avoided the possibility of misleading results obtained by the use of cell line adapted isolates.

3.5.2 Neutralization of Viruses from the Natural History cohort in rural SW Uganda

As it proved difficult to obtain follow-up samples and clinical data from persons who had attended the UVRI clinic, later work was restricted to primary viruses and plasma obtained in 1994/5, through the MRC HIV Repository, from the Natural History cohort in rural SW Uganda.

3.5.2.1 Neutralization of Primary Isolates from the Natural History cohort in rural SW Uganda

I investigated the neutralization of ten of the isolates from the Natural History cohort with autologous and heterologous plasma. Representative neutralization v plasma dilution plots are shown in detail in Figures 16i, 16ii, 16iii, 16iv and 16v and the data from this series of experiments and the repeat assays are summarized in Table 10. Plasma that were assayed but did not neutralize the viruses have not been plotted on the neutralization charts (Fig.16). Table 10 also shows the viral genotype (based on *gag* and *env* sequences). Due to problems in growing some isolates and the small volume of plasma available for 3029, it was not possible to test all of the plasma with all of the corresponding viruses to produce a complete "chequer board" analysis. All the neutralization assays illustrated in figure 16 were replicated and all the data shown were reproducible. The reason for showing the neutralization charts (Fig. 16) as well as summarizing the data in table 10 was because of the variation in the shapes of the neutralization curves, and the consequent difficulties in their interpretation. These difficulties in interpretation include the allocation of 75% neutralization titres to shallow curves that vary little with antisera dilution across the range

Figure 16i to 16v. Neutralization of primary isolates of HIV-1 in PBMC. The graphs show the concentration of p24 antigen produced in the PBMC cultures plotted against the reciprocal of the dilution of the neutralizing plasma.

Figure 16i

a/ Isolate 3004

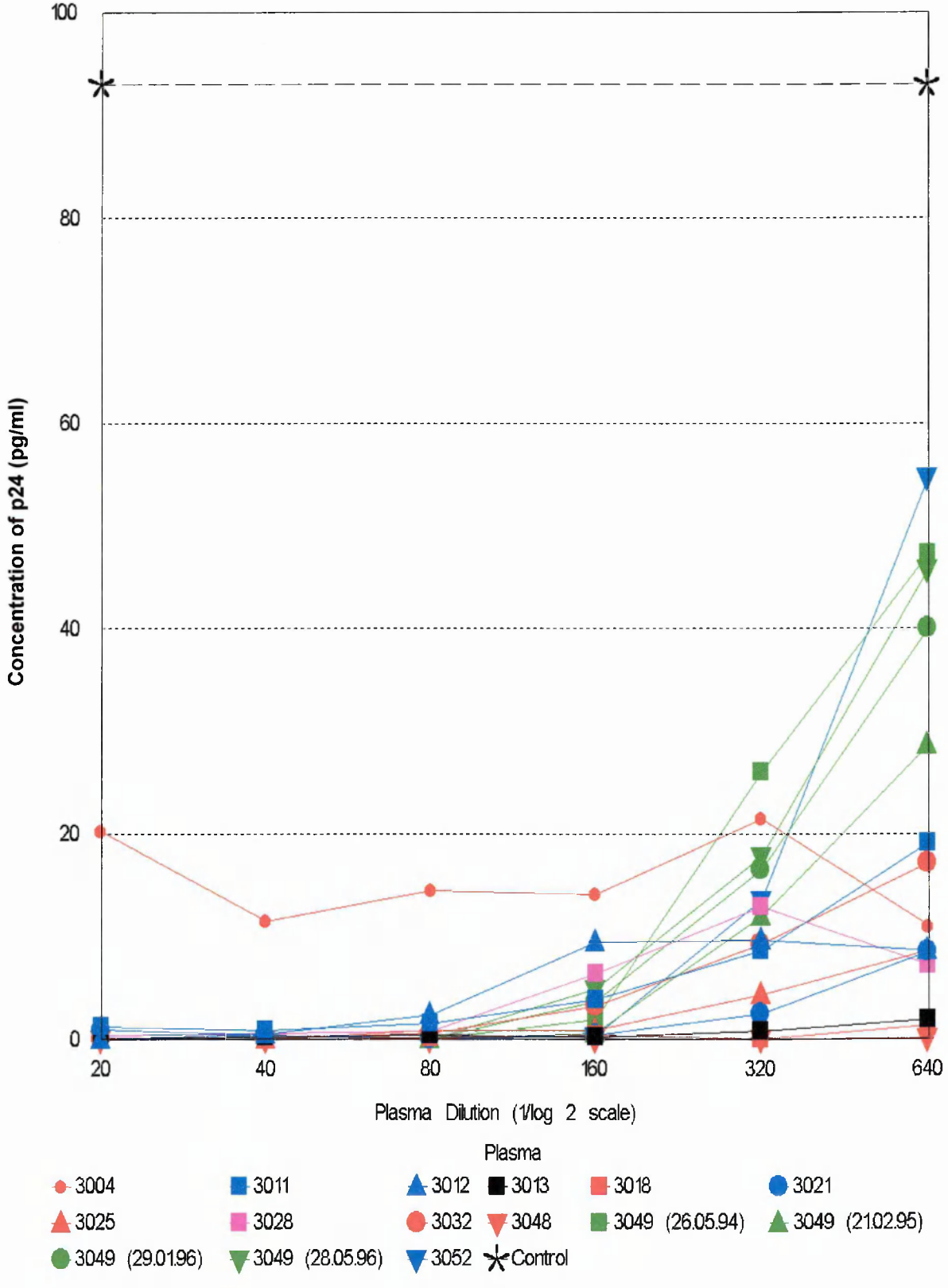


Figure 16i

b/ Isolate 3050

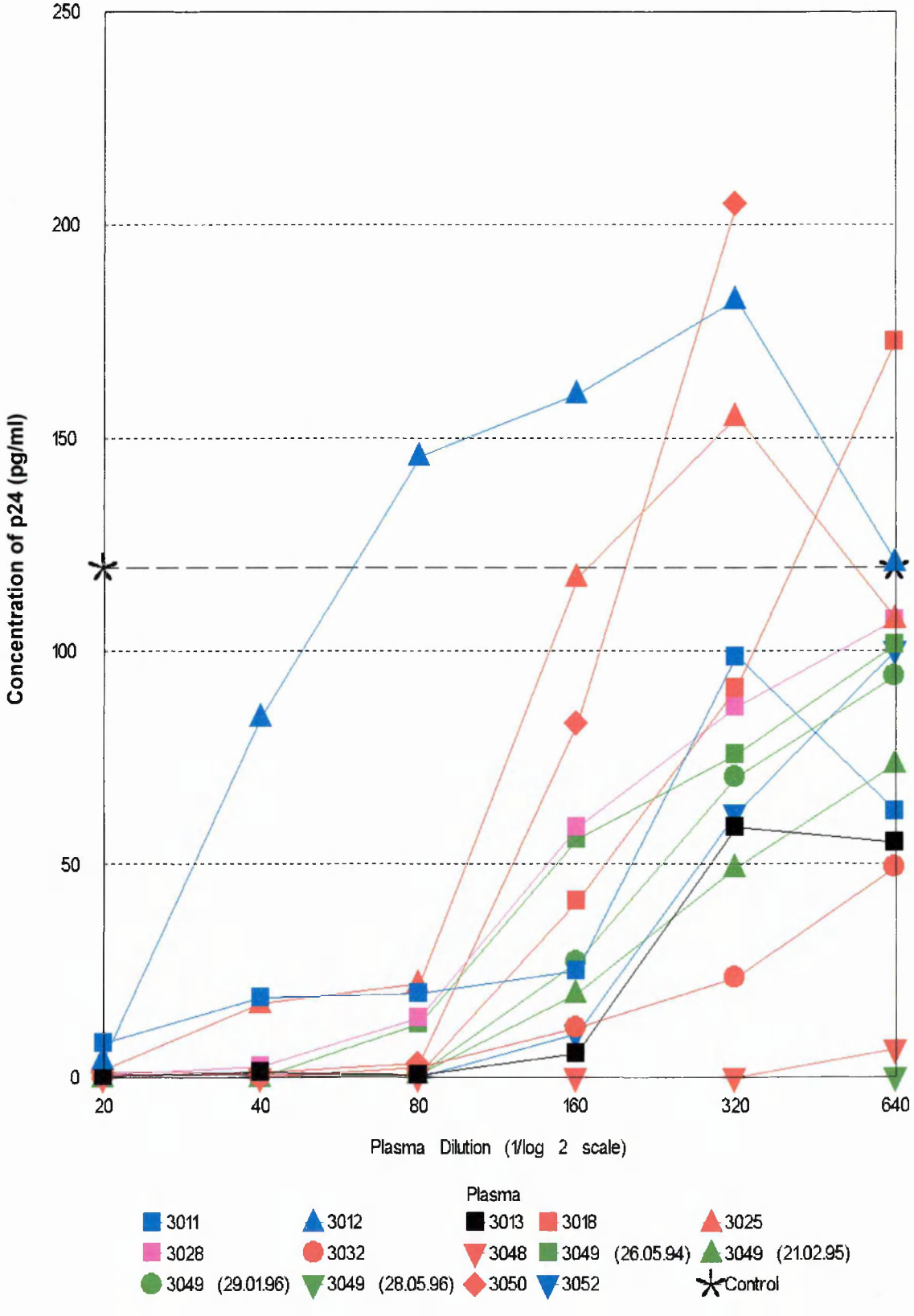


Figure 16i

c/ Isolate 3052

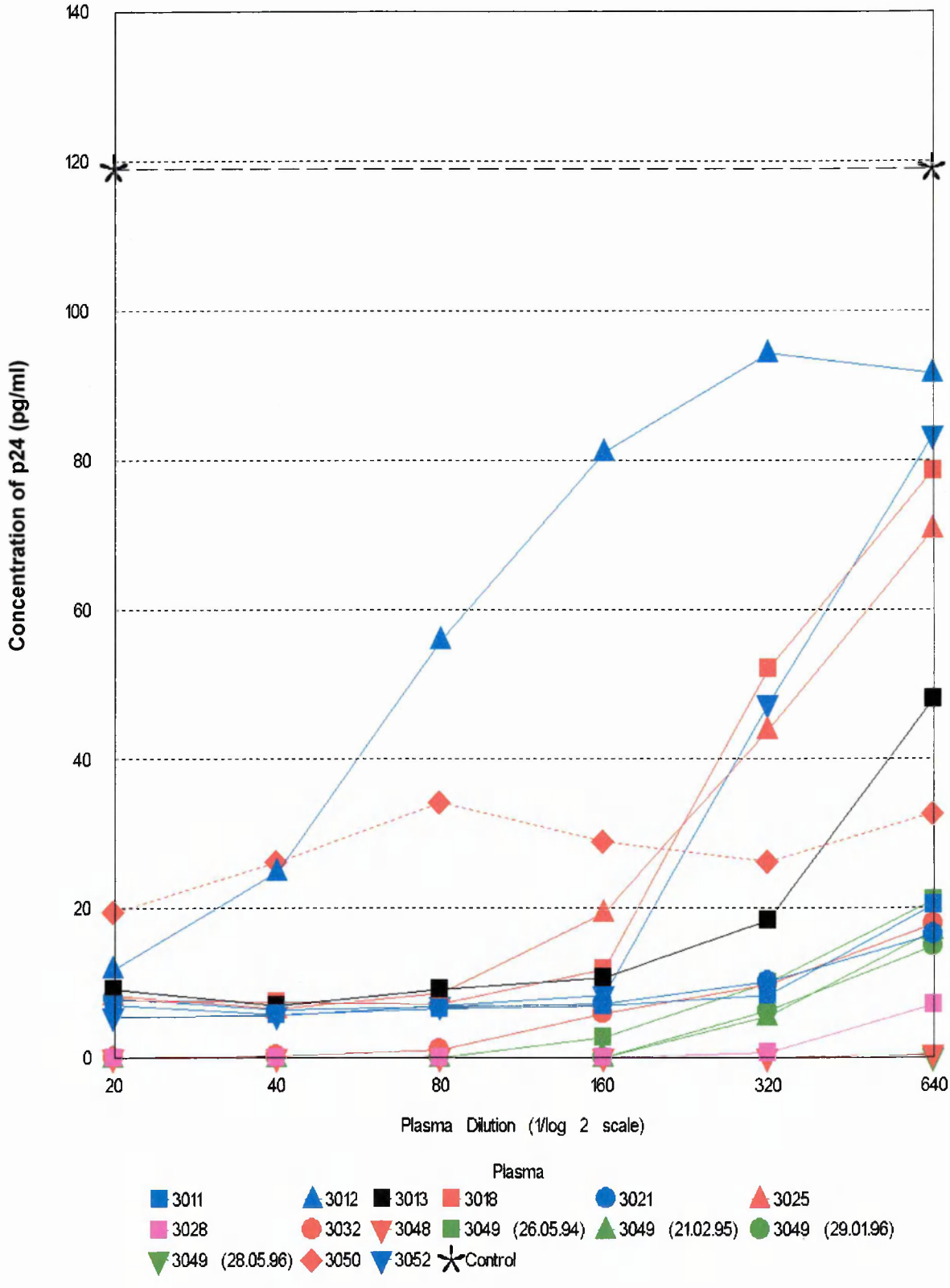


Figure 16ii

a/ Isolate 3032

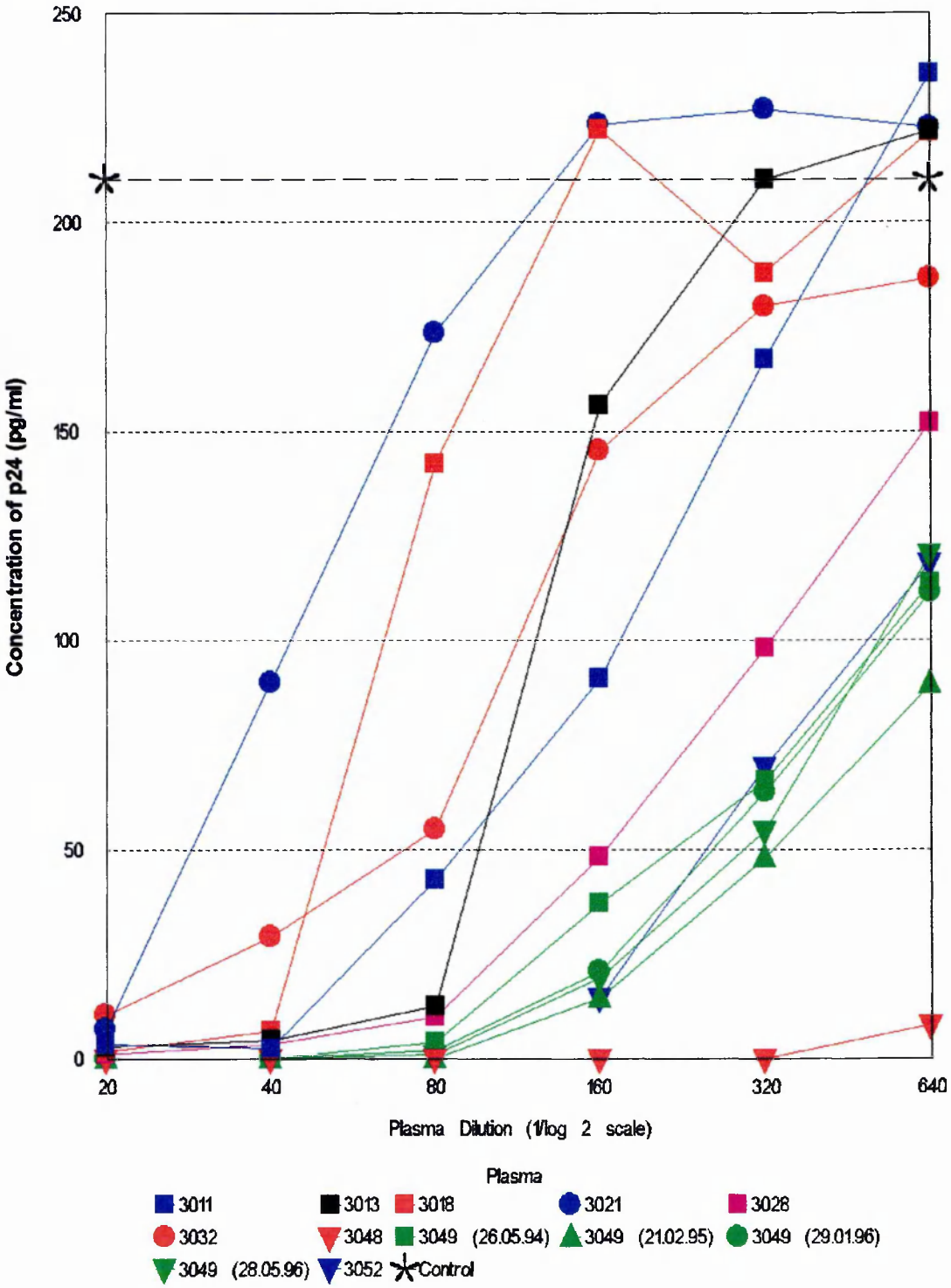


Figure 16ii

b/ Isolate 3011

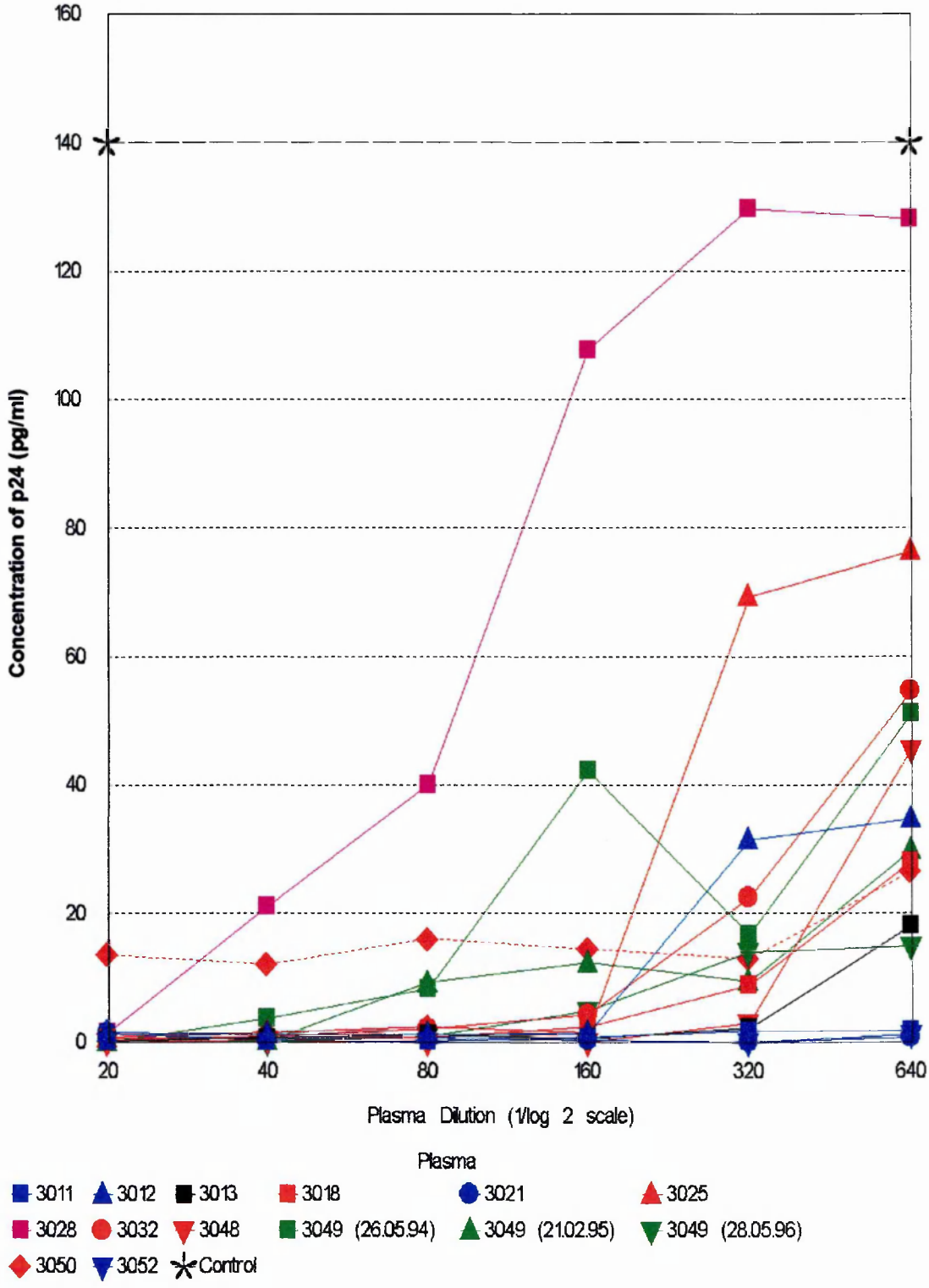


Figure 16ii

c/ Isolate 3025

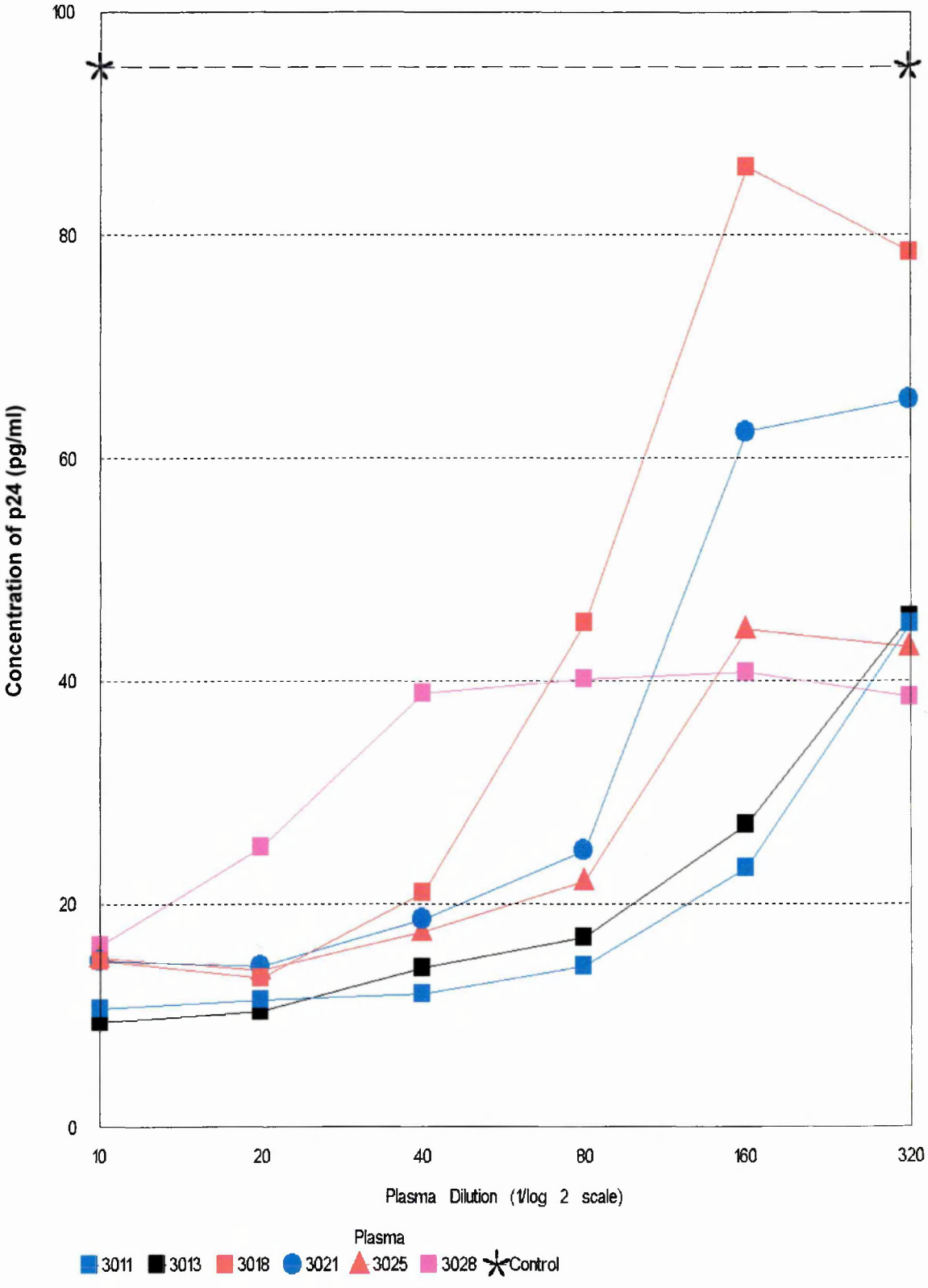


Figure 16iii

a/ Isolate 3028

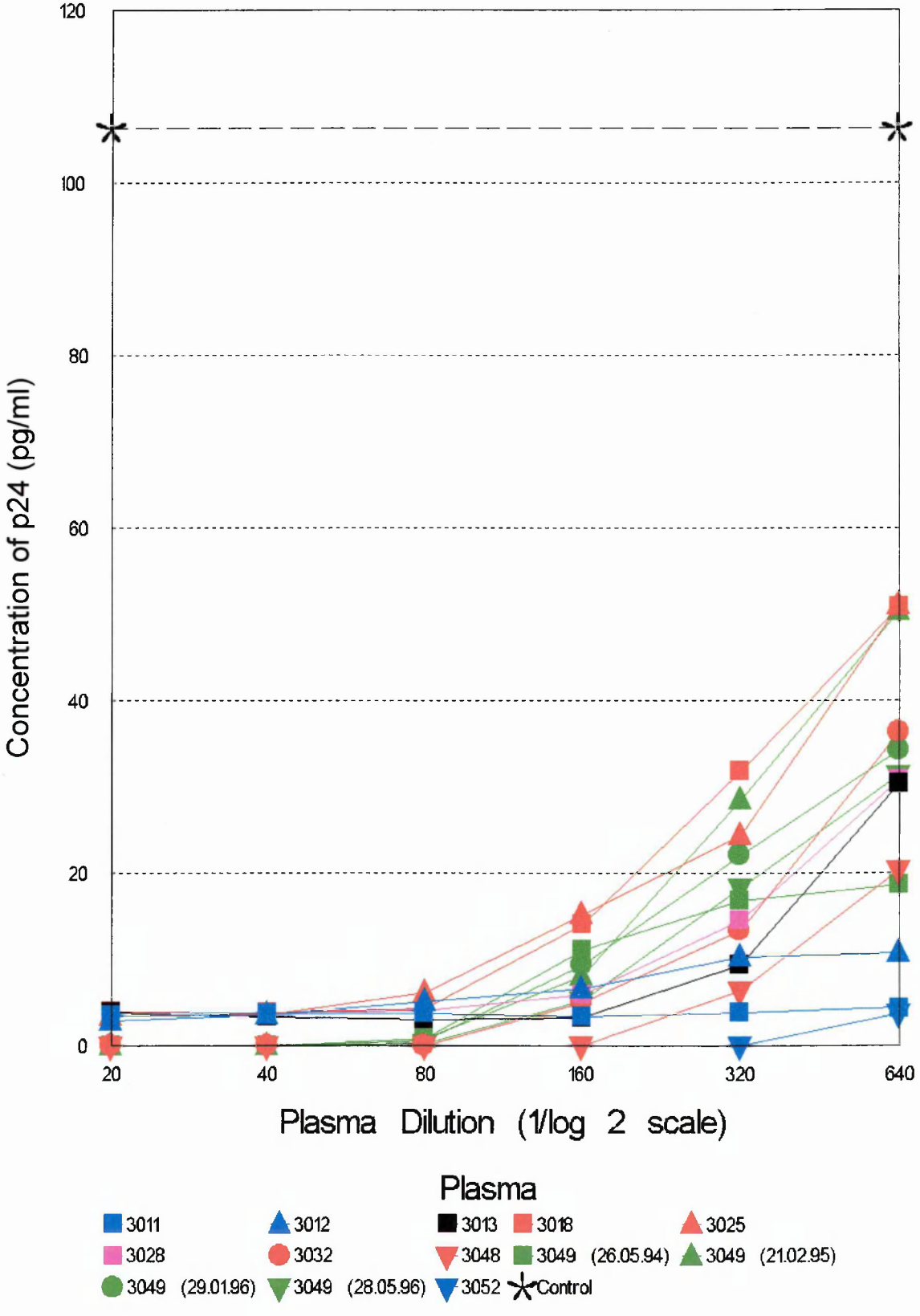


Figure 16iii

b/ Isolate 3048

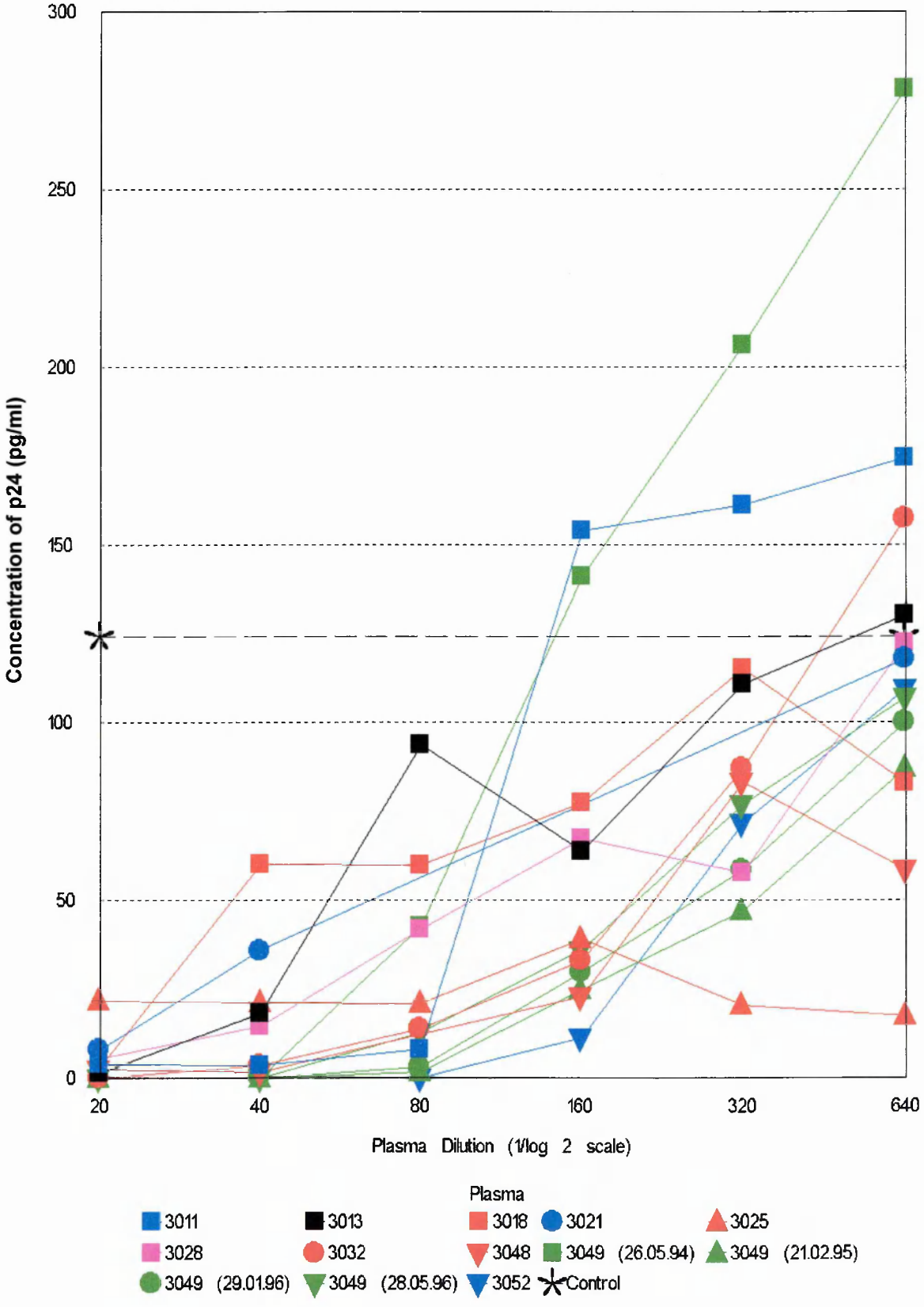


Figure 16iv

a/ Isolate 3029 (22.02.94)

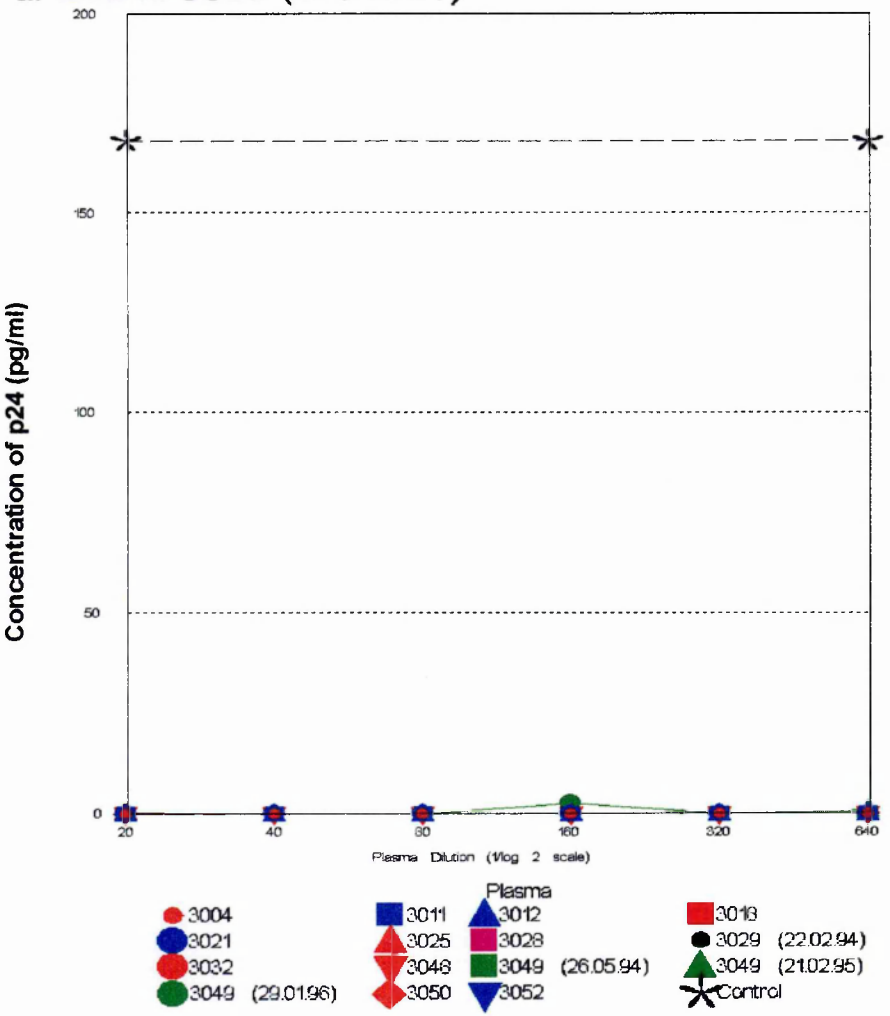


Figure 16 iv

b/ Isolate 3029 (06.06.96)

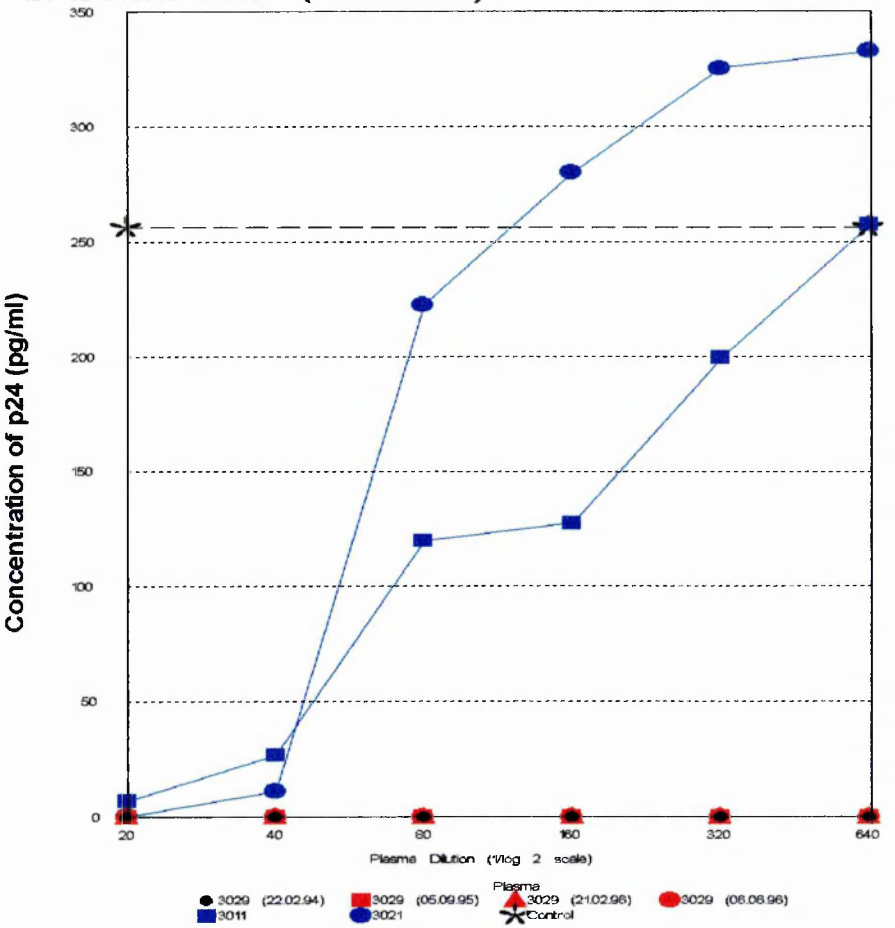


Figure 16v

a/ Isolate 3049 (26.05.94)

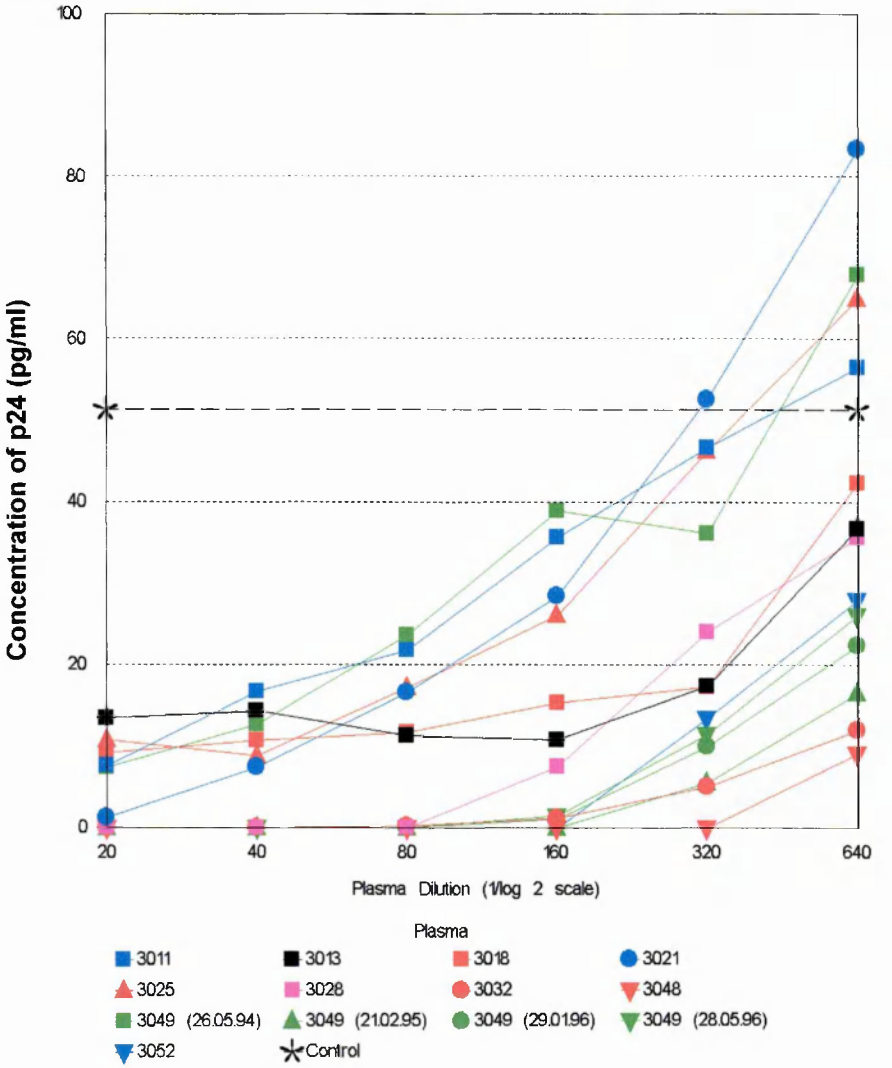


Figure 16v

b/ Isolate 3049 (28.05.96)

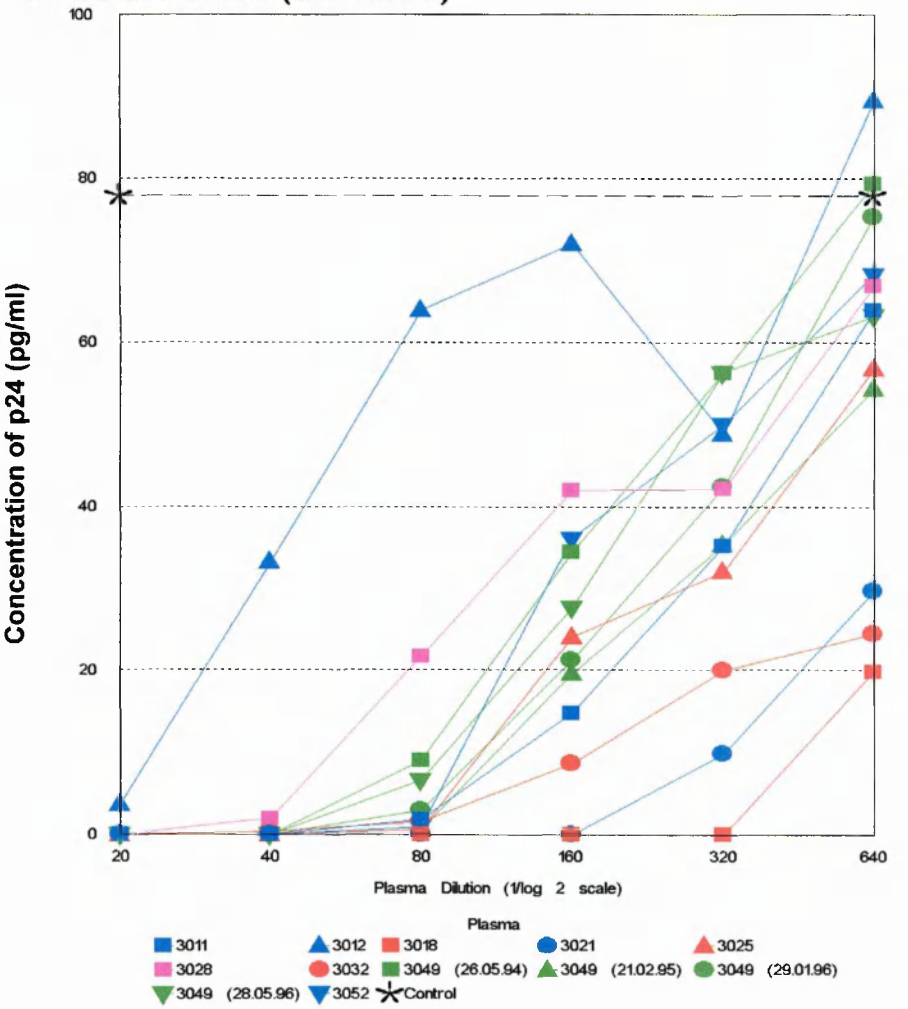


TABLE 10. NEUTRALIZATION OF UGANDAN HIV-1 ISOLATES IN PBMC CULTURES.

Plasma (Subtype)	Virus Isolate (Subtype)									
	3004 (D)	3011 (A)	3025 (D)	3028 (A/D)*	3029 (D)	3032 (D)	3048 (D)	3049 (D)	3050 (D)	3052 (A)
3004 (D)	320	-	-	-	>640	-	-	-	-	-
3011 (A)	>640	>640	160	>640	>640	100	80	30	160	>640
3012 (A)	>640	640	-	>640	>640	-	-	-	20	40
3013 (C)	>640	>640	120	600	ND	120	40	200	240	480
3018 (D)	>640	>640	40	240	>640	50	30	80	120	200
3021 (A)	>640	>640	60	-	>640	30	30	60	-	>640
3025 (D)	>640	240	80	320	>640	-	>640	60	80	200
3028 (A/D)*	>640	40	15	480	>640	160	60	200	100	>640
3029 (D)	ND	ND	ND	ND	>640	ND	ND	ND	ND	ND
3032 (D)	>640	480	ND	480	>640	80	160	640	320	>640
3048 (D)	>640	480	ND	>640	>640	>640	160	>640	>640	>640
3049 (D)	320	480	ND	>640	>640	240	60	40	100	>640
3050 (D)	-	>640	ND	-	>640	-	-	-	100	60
3052 (A)	480	>640	ND	>640	>640	320	240	320	240	200

Figures shown as reciprocal of plasma dilution giving 75% HIV-1 inhibition as determined by p24 detection.
ND = Assay not done. - = Inhibition less than 75%. * = *gag* subtype A and *env* subtype D. Shading indicates autologous virus isolate and plasma.

tested in these assays. Samples were obtained on more than one occasion for 3029 and 3049; throughout this thesis the first sample collected is implied, unless otherwise indicated by a date in brackets after the sample number.

The salient points to emerge from these data are:
(i) with two exceptions, all the plasma neutralized most of the viruses, in many cases with high neutralizing titres.
(ii) neutralization was not subtype (clade) specific.
(iii) some of the viruses were very susceptible to neutralization, others were less sensitive.
(iv) all the viruses tested were neutralized by the autologous plasma.
(v) some of the plasma showed virus enhancing activities.

The points listed above are illustrated in detail by the data shown in Fig.s 16i-v and Table 10.

(i) Plasma have broadly specific neutralizing activities:
With the exception of plasma 3004 and 3050, which neutralized only 2 out of 10 or 4 out of 9 viruses, respectively, all the plasma neutralized most of the viruses tested. For example, as shown in Table 10 and Fig.s 16i-v, plasma 3048 strongly neutralized all the isolates tested. This plasma neutralized the 3 isolates shown in Fig.16i.a/, b/ and c/ by more than 95% at a dilution of 1:640 and neutralized 7/9 isolates with a neutralization titre of 640 and isolate 3011 with a titre of 480 but had a lower titre of 160 against the autologous virus (Fig.16iii.b/). As shown in Fig.s 16i-v, plasma 3052 also had very high neutralization titres (200 to >640) against the 9 viruses tested and several other plasma, e.g. 3011, 3013, 3028 and 3032, had high titres against most of the viruses.
The presence of anti-p24 antibodies (Mascola & Burke, 1993) and of non-antibody factors in antisera including α interferon, cytokines, chemokines (Rantes, Mip-1α, Mip-1β, SDF-1) and CD8+ T cell antiviral factor can inhibit HIV-1 growth in a non-specific manner (Burrer *et al.*, 2001). My results showed

that many (15 / 23) of the antisera did not neutralize some of the viruses, these antisera showed no evidence of the presence of anti-p24 antibodies nor non-antibody factors which would be expected to inhibit the growth of all the viruses with which they were incubated. Many of the non-antibody factors mentioned above could be removed by 10KDal to 50KDal dialysis of antiserum prior to assessment in neutralization assays. IgG depletion assays would determine any contribution to the inhibition of viral growth by other factors.

(ii) Most of the plasma had cross-clade neutralizing activity:

The neutralization experiments with the isolates from the Natural History cohort in rural SW Uganda were done using 2 subtype A viruses and 8 *env* subtype D viruses. Both subtype A and D isolates were neutralized by plasma obtained from persons infected with *env* subtype A, C and D viruses. This phenomenon is illustrated in several of the figures, including Figure 16i.a/ which shows the neutralization of isolate 3004 (subtype D) and Figure 16ii.b/ which shows the neutralization of isolate 3011 (subtype A). Two plasma did not demonstrate cross-clade neutralizing activity. Plasma 3004 only neutalized 2 isolates 3004 and 3029 - both subtype D. Plasma 3029 was only available in a very small volume, so this plasma was only assayed against the autologous isolate (and sequential autologous isolate).

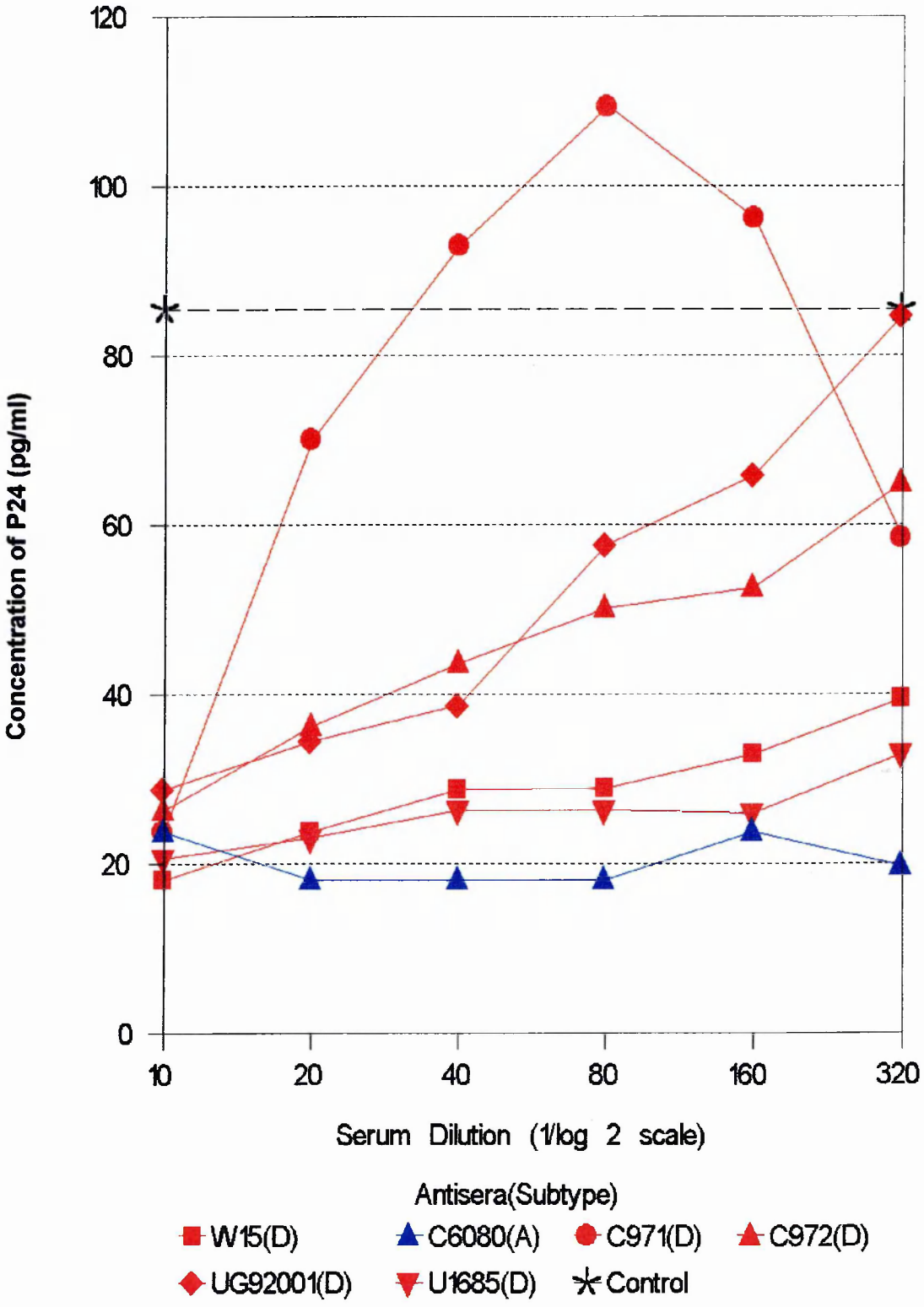
Similar results were obtained with viruses and sera from the UVRI clinic at Entebbe. Thus, figure 17 which shows the neutralization of subtype D isolate UG92035 by sera obtained from persons infected with a subtype A virus (C6080) or subtype D viruses (e.g., U1685 and W15). The back-adapted HIV-1_{MN} was also neutralized by sera from both subtype A and D virus-infected persons (Table 8 and Figure 15.d/).

(iii) Viruses differed in their sensitivity to neutralization:

Isolate 3029(22.02.94) was especially sensitive to neutralization: all 13 of the plasma used reduced p24 antigen production by more than 95% (Fig.16iv.a/). Other very sensitive viruses included 3004 (Fig.16i.a/) neutralized at high

Figure 17. Cross-clade neutralization and antibody enhancement of virus growth of primary isolate HIV-$1_{UG92035}$ (subtype D) in PBMC by antisera from persons infected with subtype A and D viruses. The graph shows the concentration of p24 antigen produced in the cultures plotted against the reciprocal of the dilution of the neutralizing antiserum.

Figure 17


120
100
80
60
40
20
0
Concentration of P24 (pg/ml)
10
20
40
80
160
320
Serum Dilution (1/log 2 scale)
Antisera(Subtype)
W15(D)
C6080(A)
C971(D)
C972(D)
UG92001(D)
U1685(D)
Control

dilutions by 12 out of 13 plasma, and 3052, neutralized by 12 out of 13 plasma (Fig.16i.c/), nearly all at high dilutions. Isolates 3032 (Fig.16ii.a/) and 3048 (Fig.16iii.b/) were less sensitive, being neutralized by 9/13 and 10/13 plasma respectively: many of the plasma had a much lower range of titres against these viruses.

(iv) Viruses were neutralized by autologous plasma:

All the isolates were neutralized to varying degrees, with titres ranging from 40 to greater than 640, by their autologous plasma (see the shaded areas of Table 10). These results contrasted with some of the data obtained using viruses isolated from the UVRI clinic blood samples, two of which (C24 and UG92001) were not neutralized by their autologous sera (see the shaded areas of Table 8).

(V) Virus enhancing activities:

Figure 17 and Figure 15f/ show examples of antibody enhancement of the growth of the primary isolates UG92035 and C971 (both from Entebbe) by antiserum C971 (also from Entebbe). Antibody enhancement was also found in four neutralization experiments involving viruses from the Natural History cohort in rural SW Uganda: isolate 3029(06.06.96) with plasma 3021 (Fig.16iv.b/), isolate 3048 with plasma 3011 and 3049(26.05.94) (Fig.16iii.b/), isolate 3049(26.05.94) with plasma 3011, 3021, 3025 and 3049(26.05.94) (Fig.16v.a/) and isolate 3050 with plasma 3012, 3018, 3025 and 3050 (Fig.16i.b/). Five of the eight plasma (C971, 3011, 3021, 3025 and 3049(26.05.94)) were involved in the enhancement of the growth of more than one primary isolate. Although this enhancement of virus growth was not investigated it may have been caused by the action of a component of the growth medium on the specific virus-antibody complexes (Booth, 1977), or enhancing antibodies which are induced by conformational epitopes in the carboxyl half of the gp120 molecule which may only be able to bind to certain isolates (Takeda *et al.*, 1992).

3.5.2.2 Neutralization of "Follow-up" Viruses from the Natural History cohort in rural SW Uganda

I produced sequential isolates of the 3029 and 3049 viruses from blood samples which I obtained 2 years after those used to get the original isolates. The two isolates of 3029 were made 20 and 48 months, and the two isolates of 3049 were made 6 and 30 months, after the first positive tests for HIV. The neutralization curves of the earlier and later isolates of 3029 and 3049 are compared in Figs 16iv and 16v and the data are summarized in Table 11. Several sequential serum samples for the two isolates were also included in these experiments. The earlier 3029 isolate was neutralized at >1:640 dilution by all the antisera tested, including the 4 autologous sera, but although the later isolate was still sensitive to the autologous sera, it was not neutralized by two heterologous sera (3025 and 3028) and was much more resistant to two other heterologous sera (3011 and 3021).

Differences in the neutralization titres of the antisera were also seen between the two isolates of 3049. Six antisera had higher titres against the later than against the earlier isolate and six antisera, including three of the sequential autologous antisera, had lower neutralization titres against the later isolate. The later (sequential) 3049 antisera had much high neutralization titres against the earlier isolate of 3049 than the antiserum taken at the same time as the first virus isolation; this shows a changed or increased immune response to the virus during the two years during which the sequential samples were taken.

TABLE 11. NEUTRALIZATION OF SEQUENTIAL UGANDAN ISOLATES 3029 AND 3049 IN PBMC CULTURES.

Plasma (Subtype)	Isolate (Subtype)	
	3029/94 (D) 22.02.94	3029/96 (D) 06.06.96
3011 (A)	>640	40
3021 (A)	>640	40
3025 (D)	>640	-
3028 (A/D)	>640	-
3029/22.02.94 (D)	>640	640
3029/05.09.95 (D)	>640	640
3029/21.02.96 (D)	>640	640
3029/06.06.96 (D)	>640	640

Plasma (Subtype)	Isolate (Subtype)	
	3049/94 (D) 26.05.94	3049/96 (D) 28.05.96
3004 (D)	-	-
3011 (A)	30	160
3012 (A)	-	30
3018 (D)	80	640
3021 (A)	60	480
3025 (D)	70	140
3028 (A/D)	200	60
3032 (D)	640	300
3052 (A)	320	120
3049/26.05.94 (D)	40	120
3049/21.02.95 (D)	540	160
3049/29.01.96 (D)	320	160
3049/28.05.96 (D)	320	120

Figures shown as reciprocal of plasma dilution giving 75% HIV-1 inhibition as determined by p24 detection.
- = Inhibition less than 75%. Shading indicates autologous virus isolate and plasma.

3.6 Correlation of Serological and Neutralization Data

Comparison of serological data obtained by reacting human sera with V3 loop peptides with the corresponding virus neutralization patterns indicated that there was no clear cut relationship between the two types of reactivity. For example, considering the isolates from Kampala and UVRI clinic samples:
several plasma, including C971, C972, W15 and UG92001, did not react with the U31 V3 loop peptide (Table 2) but neutralized the U31 primary isolate, with neutralization titres ranging from 30 to 120 (Table 8), not significantly higher than the titre (160) obtained with plasma U1685, which reacted strongly with the U31 peptide. Further, plasma UG92035, which did not react with any of the V3 loop peptides tested, neutralized 6 out of the 8 viruses tested.

Similar data were obtained with plasma/virus samples obtained from the Natural History cohort in rural SW Uganda. For example, plasma from the subtype D virus 3004 reacted with whole V3 loop peptides of MN, UG A, UG D and U31, but only neutralized the autologous isolate (Table 10), indicating that there was no correlation between serotype and neutralization phenotype.
Plasma 3013, from a subtype C virus reacted with peptides of UG A and UG D and neutralized isolates of these subtypes equally strongly indicating that the genotype (C) did not correlate with the serotype and neutralization phenotype.
Plasma 3048 reacted with the MN and UG D peptides, but not with the UG A peptide, but neutralized both subtype A and D viruses, with equally high titres (mostly >640).
Hence, much of my data indicates that, for many of the plasma/virus interactions, at least part of the neutralizing activity was not directed against the linear V3 loop epitopes.

3.7 Antibodies Raised against Linear V3 loop Epitopes in Rabbits

A major problem in attempting to correlate viral genotype or phenotype with serological properties, including neutralization specificities and titres, is that the sequences of the primary immunogens including, for example, neutralization

epitopes, cannot be established, except possibly for very recently infected persons - who are almost impossible to find in an African setting. One way of addressing this problem is to study antisera from animals immunized with carefully defined epitopes. Therefore, I investigated the ability of linear V3 loop epitopes to induce neutralizing antibodies in rabbits and then attempted to correlate the immune responses to the known sequences.

The whole V3 loop peptides used as immunogens were obtained as relatively highly pure products (>90% purity, as assessed by nuclear magnetic resonance). As detailed in Section 2.6, a portion of each peptide was coupled to a carrier protein (KLH) and the rabbits were immunized either with coupled or uncoupled peptides. The reactivities of the sera were studied in serological assays with the V3 loop peptides used as immunogens and their neutralizing activities determined using 8 primary isolates from the Kampala/Entebbe area and the back-adapted HIV-1_{MN}.

3.7.1 Serological Studies on Antibodies Raised against Whole V3 loop Peptides in Rabbits

The reactivities of the rabbit antisera to the MN, UG A, UG D and U31 whole V3 loop peptides are shown in Table 12.a. Twelve of the 16 rabbits gave an immune response, with all 12 reacting with the inoculum peptide. Five of the 12 antisera were mono-specific, reacting only with the inoculum peptide (U31 or UG A): four reacted with two peptides, two antisera reacted with three peptides and one antiserum reacted with all four peptides.

With the exception of antisera 704 and 719, the cross-reactive antisera responded most strongly towards the inoculum peptide.

Vaccination with the MN peptide gave the highest titre antisera, which also had the most cross-reactivity and vaccination with the U31 peptide gave the most specific antisera, which reacted only with the U31 peptide.

Table 12.b is a summary of the data shown in table 12.a, which shows the mean titres for the reactive sera. The shaded areas of the table illustrate binding to the

TABLE 12.a. REACTIVITY OF RABBIT ANTISERA RAISED TO V3 LOOP PEPTIDES.

RABBIT SERA	INOCULA	PEPTIDES USED IN ASSAYS			
		MN	UG A	UG D	U31
701	MN	5400	1200	-	-
705	MN	6000	4200	5400	-
719	MN/KLH	2430	3600	3600	200
723	MN/KLH	3600	1600	1200	-
700	UG A	-	-	-	-
708	UG A	-	600	200	-
704	UG A/KLH	1800	1200	-	-
724	UG A/KLH	-	-	-	-
707	UG D	-	-	200	-
699	UG D	-	400	500	-
702	UG D/KLH	-	-	200	-
721	UG D/KLH	-	-	-	-
703	U31	-	-	-	2100
706	U31	-	-	-	-
722	U31/KLH	-	-	-	200
720	U31/KLH	-	-	-	1800

Titres are reciprocals of serum dilutions giving an EIA of 0.5 OD units.
- = titre < 100. KLH = Keyhole Limpet Haemagglutinin conjugate.

TABLE 12.b. SUMMARY OF THE BINDING OF RABBIT ANTISERA RAISED TO V3 LOOP SPECIFIC PEPTIDES.

INOCULA	PEPTIDES USED IN ASSAYS			
	MN	UG A	UG D	U31
MN	4400	2700	2600	200
UG A	1800	900	200	-
UG D	-	400	300	-
U31	-	-	-	1400

Titres are reciprocals of serum dilutions divided by the number of reactive sera (ie. average titre) giving an EIA of 0.5 units of absorbance.
- = titre < 100.
Shading indicates homologous inoculum and peptide.

inoculum peptide. The table shows that sera from rabbits vaccinated with the MN and UG A peptides were cross-reactive, with similar titres towards the MN and UG D peptides: however, this cross-reactivity was only partial as vaccination with the UG A peptide failed to induce UG A-reactive antibodies. Table 12.b. also shows that the UG D peptide was poorly immunogenic.

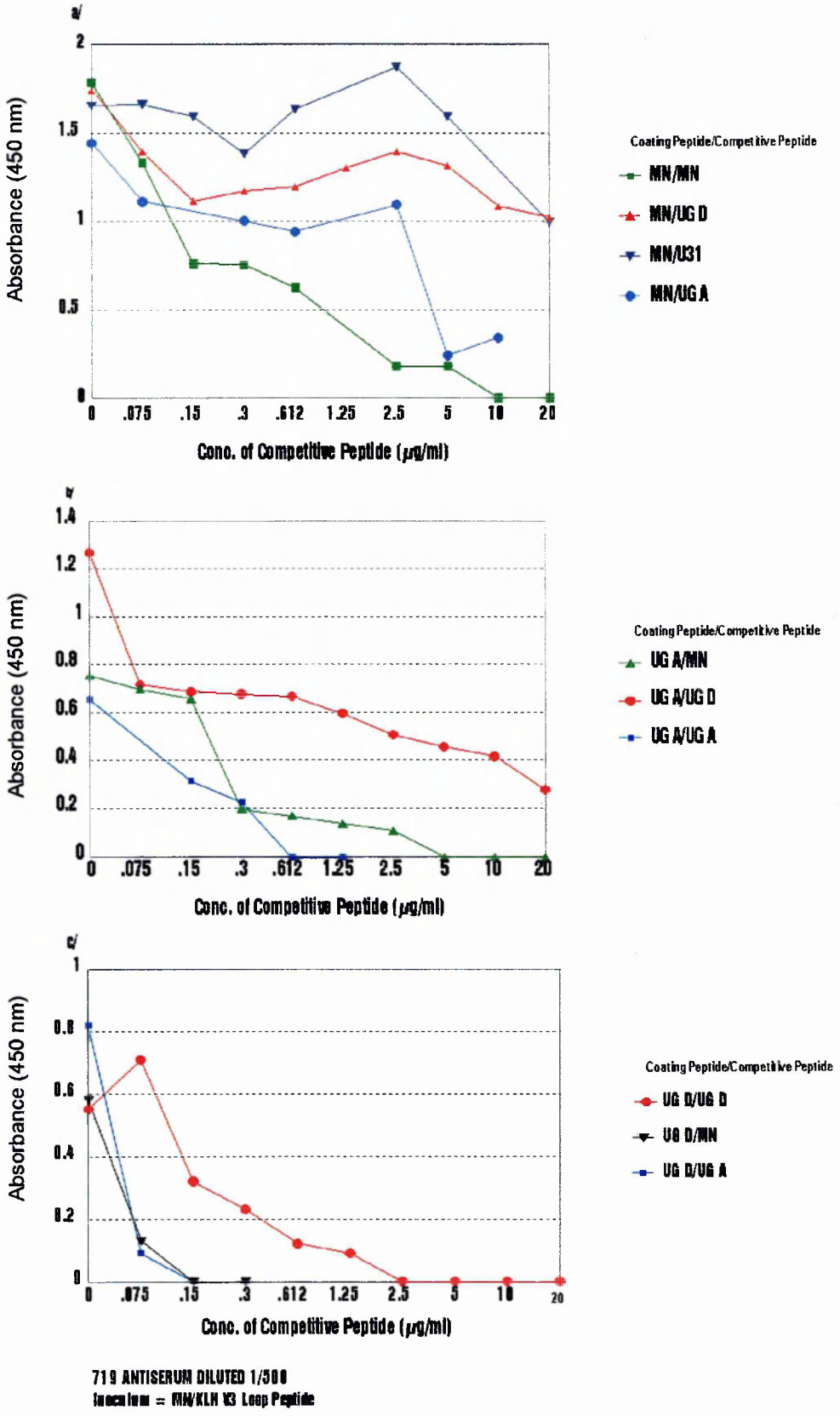
The specificities of the cross-reactive rabbit antisera were examined by competitive inhibition assays. The results for rabbit antiserum 719 at 1:500 dilution, an example of the sera raised against the MN peptide, are shown in Figure 18. As shown in Fig.18.a/ the MN peptide was the most effective competitive inhibitor of the MN-reactive antibodies present in this serum, giving 90% inhibition at 2.5μg/ml. The UG A peptide was less effective (80% inhibition at 5μg/ml) and the UG D and U31 peptides were not inhibitory.

Similarly, the UG A peptide was the most effective competitive inhibitor of the UG A reactive antibodies, giving 90% inhibition at 0.4μg/ml. The MN peptide inhibited UG A reactive antibodies by 90% at 2.5μg/ml. The UG D peptide did not inhibit UG A reactive antibodies (Fig.18.b/). Surprisingly, the UG D peptide was a poor inhibitor of the UG D reactive antibodies which, however were inhibited strongly by the MN and UG A peptides (Fig.18.c/).

These data indicated that the MN peptide induced cross-reactive antibodies which were specifically recognized by both MN and UG A peptides. The inhibition of MN-induced UG D-reactive antibodies by the MN and UG A peptides indicates that this reactivity was probably also due to antibodies directed against the MN and/or UG A epitopes. The UG D peptide appeared to be less effective as an immunogen, but was a good competitor for the UG D-reactive antibodies.

Figure 18. Competitive inhibition assays of rabbit 719 antiserum (1:500 dilution) raised to the MN V3 loop peptide. The serum was preincubated with MN, UGA, UG D and U31 peptides before measuring peptide reactivities. Each graph shows the absorbance (λ=450nm) obtained in the standard antibody binding assay plotted against the concentration of the competitive peptides.

Figure 18


a/
Absorbance (450 nm)
2
1.5
1
0.5
0
0 .075 .15 .3 .612 1.25 2.5 5 10 20
Conc. of Competitive Peptide (µg/ml)
Coating Peptide/Competitive Peptide
MN/MN
MN/UG D
MN/U31
MN/UG A
b/
Absorbance (450 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 .075 .15 .3 .612 1.25 2.5 5 10 20
Conc. of Competitive Peptide (µg/ml)
Coating Peptide/Competitive Peptide
UG A/MN
UG A/UG D
UG A/UG A
c/
Absorbance (450 nm)
1
0.8
0.6
0.4
0.2
0
0 .075 .15 .3 .612 1.25 2.5 5 10 20
Conc. of Competitive Peptide (µg/ml)
Coating Peptide/Competitive Peptide
UG D/UG D
UG D/MN
UG D/UG A
719 ANTISERUM DILUTED 1/500
Immunogen = MN/KLH 123 Loop Peptide

3.7.2 Determination of Neutralizing Antibodies in Sera from Rabbits Immunized with V3 loop Peptides

Virus neutralizing activities on PBMCs of 4 of the rabbit antisera were determined using 8 primary Ugandan isolates and the back-adapted HIV-1_{MN}; the results are summarized in Table 13. One rabbit antiserum for each inoculum peptide was selected for use in these experiments; those that reacted most strongly with the inoculum peptide in the antibody binding assays were selected. The four antisera were 705 (MN), 703 (U31), 704 (UG A) and 699 (UG D). Three of the 8 Ugandan isolates (C6080, C971 and UG93070) were not neutralized by any of the rabbit antisera, but all of the antisera neutralized at least one of the other 5 isolates assayed. All of the rabbit antisera neutralized virus isolate UG92001, but at low titres. Most human sera gave a similar pattern of low titre neutralization for this isolate and the two sets of data are compared in Figure 19. The antiserum raised against the MN peptide neutralized 4 Ugandan subtype D isolates with titres ranging from 10 to 200 and neutralized the back-adapted HIV-1_{MN} with a titre of >320. The antiserum raised to the UG A consensus peptide did not neutralize the one subtype A virus tested (C6080), but did neutralize two subtype D viruses with titres of 10 and 160 and the back-adapted HIV-1_{MN} (subtype B) with a titre of >320. The antiserum raised against the UG D consensus peptide neutralized three subtype D viruses, but not the subtype A or B viruses. The antiserum raised against the U31 peptide did not neutralize the autologous virus and had only low neutralizing activity towards one other virus. Isolates C6080, C971 and UG93070 were not neutralized by any of the raised antisera, however in previous neutralization assays with human antisera both C971 and UG93070 were neutralized - C971 to titres of >320 by some antisera (See Table 8).

TABLE 13. NEUTRALIZATION OF UGANDAN ISOLATES OF HIV-1 AND HIV-1_{MN} BY RABBIT ANTISERA RAISED TO V3 LOOP PEPTIDES.

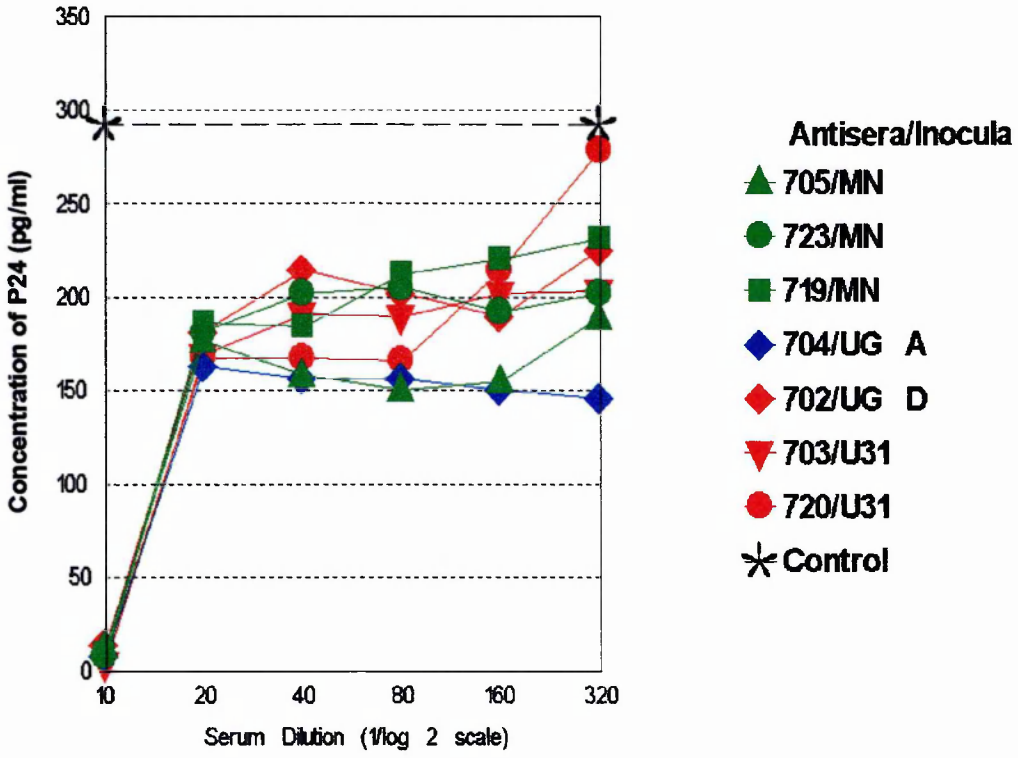
SERUM/ INOCULA	VIRUS ISOLATE (SUBTYPE)								
	C6080 (A) SI	W15 (D) SI	UG92001 (D) SI	MO47 (D) NSI	C971 (D) NSI	U31 (D) SI	UG92035 (D) SI	UG93070 SI	MN* (B) SI
705/MN	-	40	15	160	-	-	200	-	>320
704/UG A	-	-	15	-	-	-	160	-	>320
699/UG D	-	-	15	80	-	ND	30	-	-
703/U31	-	-	15	-	-	-	-	-	-

* = non-Ugandan. SI = Syncytium inducing. NSI = Non-syncytium inducing.
Figures shown as reciprocal of antiserum dilution giving 75% HIV-1 inhibition as determined by p24 detection.
ND = Assay not done. - = Inhibition less than 75%.
Shading indicates cross-clade neutralization.

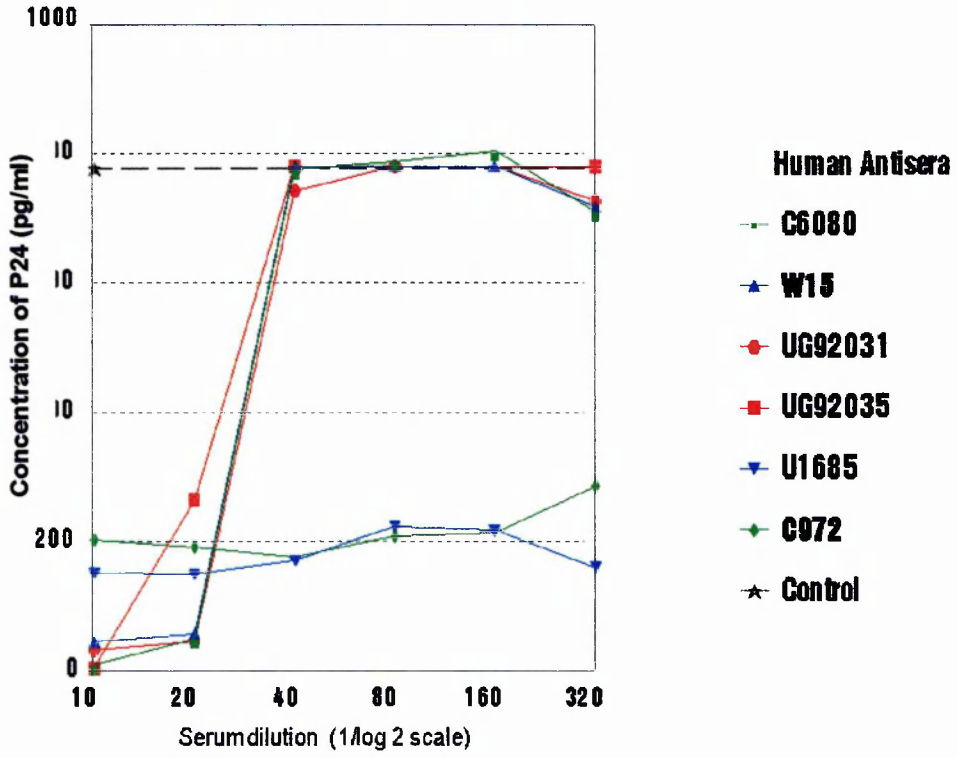
Figure 19. Neutralization of primary isolate HIV-$1_{UG92001}$ by rabbit antisera (19.a/) and by human antisera (19.b/). The graphs show the concentration of p24 Ag against the reciprocal of the dilution of the neutralizing antiserum/plasma.

Figure 19

a/


350
300
250
200
150
100
50
0
Concentration of P24 (pg/ml)
10
20
40
80
160
320
Serum Dilution (1/log 2 scale)
Antisera/Inocula
705/MN
723/MN
719/MN
704/UG A
702/UG D
703/U31
720/U31
Control

b/


1000
10
10
10
200
0
Concentration of P24 (pg/ml)
10
20
40
80
160
320
Serum dilution (1/log 2 scale)
Human Antisera
C6080
W15
UG92031
UG92035
U1685
C972
Control

3.7.3 Correlation of Serological Reactivities and Specificities of Virus Neutralization by Antisera Raised in Rabbits

There have been several reports that the V3 loop region acts as the PND in several (laboratory adapted) viruses and peptides have been used to induce specific neutralizing antibodies (Page *et al.*, 1991; Wagner *et al.*, 1992; Keller *et al.*, 1993; Tolman *et al.*, 1993). However, in this study the data shows that, with the exception of the U31 peptide, the serological responses to the peptide vaccines were very broadly-specific and a similar lack of specificity was observed in the neutralization experiments. This was shown, for example, by the neutralization of subtype D viruses as well as the back-adapted HIV-1_{MN} variant by the sera induced by the MN peptide and by the neutralization of subtype D viruses and HIV-1_{MN}, but not a subtype A virus, by the serum induced by the UG A peptide. There was, however, a very loose correlation between the breadths of the serological and neutralization responses: for example, antibodies induced by the MN peptide reacted with more peptides than the other peptide immunogens and also neutralized more viruses, possibly indicating that it was the most effective immunogen in these experiments.

3.8 Correlations between Clinical and Virus Neutralization Data

One of the main objectives of this study was to determine if the rate of disease progression in members of the Natural History cohort in rural SW Uganda could be linked with the presence of neutralizing antibodies in their plasma. This cohort is recruited from the General Population cohort, which is tested in yearly serosurveys. Hence, the Natural History cohort is ideal for this type of study as the dates of seroconversion in incident cases are known to within 12 months and details of their age, sex and home village, etc are also known. At 3-monthly intervals after recruitment, cohort members are examined clinically and their blood monitored for T cell subset numbers and ratios by FACSCOUNT analysis.

Personal, clinical and T cell subset data and the estimated dates of seroconversion for 13 persons, supplied by Dr J Whitworth and Dr D Morgan of

the MRCPA in Uganda, together with the neutralization and genotype data for the corresponding viruses, are summarised in Table 14 and Figure 20. Figure 20 shows the CD4+ and CD8+ cells per μl against time in months since seroconversion for each person. With the exception of one person (listed as 3032 in table 14) who died during the study period and whose sequential T cell subset data was not available, the data for the other subjects covers between 4 and 7 visits to the clinic over a 2-3 year period, in most cases starting about 2-3 years after their first seropositive blood sample: however, for subject 3052 this period was about 18 months. The results of the clinical examinations are shown as WHO clinical stagings, as defined in the World Health Organisation proposed clinical staging system for HIV infection and disease, a copy of which is included in Appendix V. Briefly, stage 1 is asymptomatic and stages 2 and 3 are intermediate stages in the progression to AIDS, the stage 4 disease condition. The virus neutralization data shown in Table 14 gives the neutralization titres against the autologous virus and an indication of the range of neutralizing titres against heterologous viruses from the cohort, using data summarised in Table 10. The data in Table 14 was somewhat incomplete for some of the subjects; some plasma were not tested against the autologous viruses due to low viral titres on PBMCs, and in the case of the several 3029 plasma, against heterologous viruses due to low plasma volumes. Subjects with incomplete data are shown with an "ND" in their scores.

This study included seven persons (3004, 3012, 3021, 3025, 3029, 3032 and 3048) who were recorded by early 1997 as WHO Stage 3 patients (Table 14) and who were probably progressing towards AIDS. Subject 3032 died during the course of the study after developing a very low CD4 count of 50 cells/μl. Three subjects (3021, 3029 and 3049) showed a marked decline in their CD4 counts and CD4/CD8 ratios during the study period. It was not possible to test the neutralizing activity for the autologous virus of sample 3021, obtained from a person whose CD4+ cell counts fell to a very low level (<50 cells/μl), but this serum sample neutralized 8/10 heterologous isolates with neutralization titres ranging from 30 to >640. However most of the plasma and viruses used in the

Table 14. Serum Data for Samples.

Sample (subtype)	Date of serum sample	Seroconversion Date	Autologous Neutralization Titre	Heterologous Titre Range	Heterologous isolates Neutralized / Assayed	WHO Stage
3004 (D)	18.11.93	21.04.92	320	>640	1 / 9	3
3011 (A)	18.01.94	09.09.93	>640	30 to >640	9 / 9	2
3012 (A)	18.01.94	24.03.91	ND	20 to >640	6 / 10	2 / 3
3013 (C)	18.01.94	08.09.92	ND	40 to >640	9 / 9	2
3018 (D)	18.01.94	13.06.92	ND	30 to >640	10 / 10	2
3021 (A)	22.02.94	02.07.92	ND	30 to >640	8 / 10	3
3025 (D)	22.02.94	09.01.92	80	60 to >640	8 / 9	3
3029 (D)	22.02.94	09.12.91	>640	ND	0 / 0	2 / 3
3032 (D)	13.04.94	23.04.91	80	160 to >640	8 / 8	3
3046 (D)	26.05.94	19.11.91	ND	ND	0 / 0	1 / 2
3048 (D)	26.05.94	27.01.93	160	480 to >640	8 / 8	3
3049 (D)	26.05.94	01.06.92	40	60 to >640	8 / 8	2
3052 (A)	13.07.94	06.02.93	200	240 to >640	8 / 8	2

ND = Neutralization Titre not determined.
Seroconversion dates were estimated from the time of the first positive and the time of last negative results.
WHO Stage – World Health Organisation hierarchical stage at time of examination, as defined in Appendix V.
All of the clinical data came from the MRCPA.

Figure 20. CD4+ and CD8+ Cell Counts.

For each HIV-1+ person the CD4+ and CD8+ cells/μl in the peripheral blood are plotted against time in months since seroconversion.

Each plot is labelled with the sample number. The CD4+ cells are shown in blue and the CD8+ cells are shown in red. Virus isolations are indicated on the time axis by red arrows.

Person 3032 had only one set of clinical data sampled on 12.02.94 (2 months before the virus and plasma samples were taken). At this time the CD4+ count was 50 cells/μl and CD8+ count was 914 cells/μl.

All data came from the MRCPA.

Figure 20. CD4+ and CD8+ Cell Counts

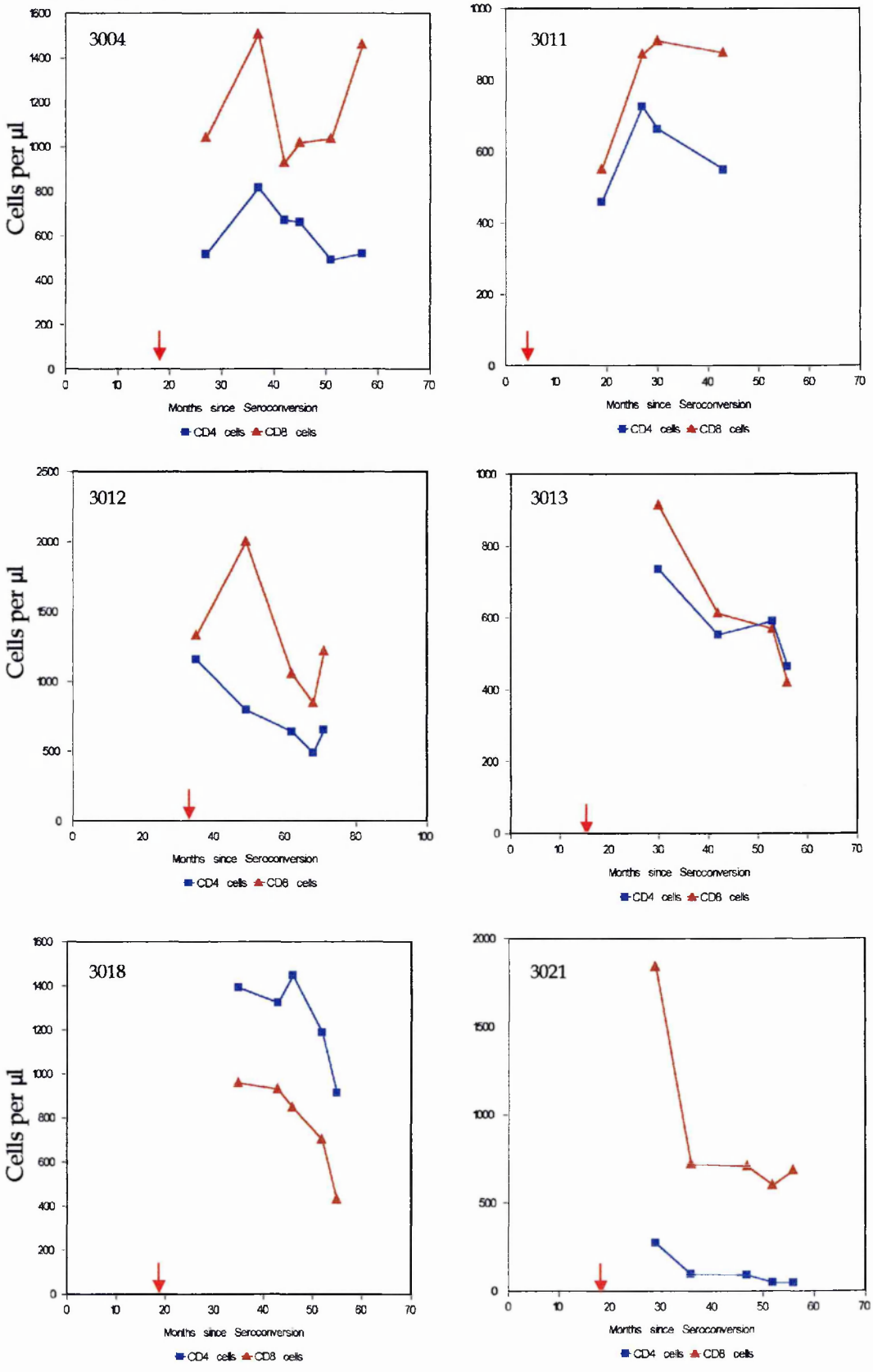
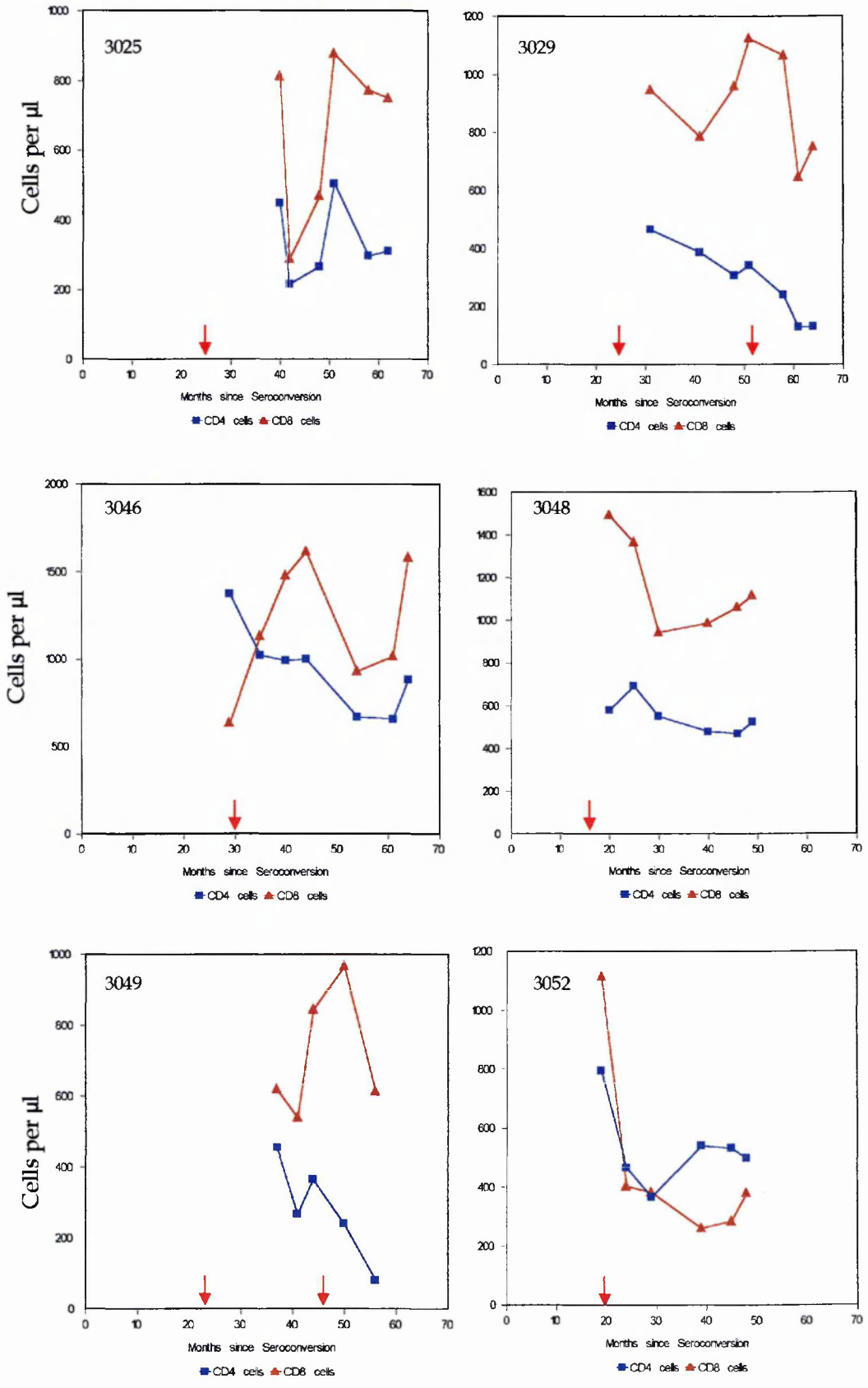


Figure 20. CD4+ and CD8+ Cell Counts



neutralization assays were obtained at an early, asymptomatic, stage. The exceptions 3029 and 3049 where sequential isolates were tested (Table 11) and plasma were obtained at about the time of CD4 cell decline.

For eight subjects (3004, 3011, 3025, 3029, 3032, 3048, 3049 and 3052) there were autologous neutralization and clinical data. These data were examined in more detail to see if there was a relationship between the autologous virus neutralizing titres and the change in the CD4+ cell count.

In four of these persons (3004, 3011, 3048 and 3052) the CD4+ cell counts remained relatively high (>500 cells/μl) over the study period. In one person (3025) the counts varied between 200 and 500 cells/μl and in 3 persons (3029, 3032 and 3049) had, or developed, low CD4+ counts (<130 cells/μl) during the study period. The samples from the 4 persons with the high CD4+ counts and CD4+: CD8+ ratios all had relatively high neutralizing antibody titres against the autologous viruses. With the exception of sample 3029, which neutralized the autologous virus at a titre of >640, the four persons with low or intermediate CD4+ counts had relatively low neutralizing titres (between 40 and 80) against their autologous viruses.

So with this small number of samples it was not possible to determine any relationship between the neutralization data and the clinical data.

Clearly, additional data would be required from earlier and later matched plasma and virus samples for a more meaningful test of the possible correlation between disease progression and the presence of neutralizing antibodies.

4. DISCUSSION

The HIV/AIDS epidemic is the major cause of adult morbidity in Uganda and the surrounding countries, although there have been indications that the seroprevalence of HIV infection may be dropping in women attending ante-natal clinics in Kampala (UNAIDS and WHO, 2002a). Further indications of a decrease in seroprevalence have come from the most recent reports from the general population cohort in rural south western Uganda (Whitworth *et al.*, 2002). The falling seroprevalence indicates that the government's AIDS awareness and sex education programmes are having an effect. However, the prevalence of HIV in young adults living in urban communities is still about 10% and the major impact of AIDS on the community is probably yet to come (UNAIDS and WHO, 2002a).

Even if the educational programmes are having some success in reducing the transmission of HIV in Uganda, there is an urgent need to develop a vaccine which will be effective against African variants of HIV. It is also important that methods should be developed to assess the effectiveness of vaccines against HIV and to monitor the spread of different virus variants or subtypes in the community. My work has been concerned with these two aspects of the HIV/AIDS epidemic in Uganda.

4.1 Serology

My first objective was to develop an enzyme-linked immunosorbent assay (ELISA) which could be used in sero-surveys to determine the distribution of different viral subtypes (clades) in different parts of Uganda and surrounding countries and to show if the pattern was changing with time. My approach was to develop a serological assay rather than more complex molecular biological methods since whereas the former can be used with plasma or sera, the latter requires a source of viral RNA or proviral DNA, which would be much less readily available in developing countries.

There were two main phases to this section of the work. Firstly, the characterization of Ugandan sera using a panel of synthetic peptides corresponding to North American and African V3 loop epitopes. This was followed by the vaccination of rabbits with whole V3 loop peptides and the analysis of antibodies induced by peptides of known structure.

Comparison of the peptide-binding specificities of HIV-positive Ugandan sera showed that there was no obvious correlation between the V3 loop sequence of a provirus and the specificity of the autologous serum. Thus, several sera reacted more strongly with the HIV-1_{MN} peptide than with peptides encoded by the autologous virus isolate. Furthermore, as the majority of the Ugandan sera were cross reactive with V3 loop peptides based on HIV-1_{MN} subtype and consensus Ugandan subtype A and D sequences, it seems unlikely that the subtype of the infecting virus could be distinguished by relatively simple peptide-based assays. It was obviously a serious problem that a very high proportion of the sera reacted strongly with the HIV-1_{MN} peptide, and also with peptides encoded by some other subtype B viruses. Other groups had previously reported that many HIV-positive African sera react strongly with V3 loop peptides encoded by subtype B viruses (Carrow *et al.*, 1991; Warren *et al.*, 1992; Blomberg *et al.*, 1993). However, with few exceptions, the majority of Africans are infected with viruses in other subtypes. Several hypotheses have been put forward to account for this paradox. A possible explanation is that most people were infected with a virus with an HIV-1_{MN}-like V3 loop epitope which subsequently evolved to a virus with a different epitope, similar to either the Ugandan subtype A or D consensus sequences. However, no HIV-1_{MN}-like viruses have been isolated from infected Ugandans and several studies (Oram *et al.*, 1991; Albert *et al.*, 1992; WHO network for HIV isolation and characterization, 1994) have shown that the amino acid sequences around the V3 loop apex of most Ugandan variants are not similar to that of HIV-1_{MN}. An

alternative explanation is that most variants induce antibodies which cross-react with a number of peptides, including that of HIV-1_{MN}.

Kohler *et al.* (1992) proposed a concept of "clonal dominance" to account for the restricted B-cell response to V3 loop epitopes described by Goudsmit *et al.* (1989) and Zwart *et al.* (1992). They proposed that during the primary viraemic stage of infection the epitope presented by the genotypically homogeneous fast-replicating viral population saturates the antigen presenting cells in the germinal centres; subsequent genetic variants contain cross-reactive dominant epitopes which re-boost the primary response. They proposed that this led to "repertoire freeze" which limited the recruitment or maturation of other uncommitted B-cells to other variants of the epitope (Kohler *et al.*, 1992). The high degree of sequence diversity of the V3 loop region of the gp120 (Oram *et al.*, 1991; Albert *et al.*, 1992; Myers *et al.*, 1993) within the provirus populations of infected Africans leads to the production of antibodies with broad, cross-reactive V3 loop peptide binding patterns. Within an infected individual evolution of the provirus V3 loop epitopes may lead to a further broadening of the specificities of V3-loop reactive antibodies (as seen in the AIDS patients in this study). This may result in the production of antibodies reactive towards a very diverse range of V3 loop epitopes, which in some cases, do not cross-react with the epitopes of the initial infecting virus population. Formation of these antibodies may well be due to re-boosting of the original B cell clones, primed during seroconversion to initial HIV infection rather than to the recruitment of uncommitted B cells.

I have shown that it is possible to separate, by affinity chromatography, antibodies which react with different V3 loop peptides. This indicates that many infected Ugandans produce antibodies against distinct variants of the V3 loop epitope. For example, HIV-1_{MN} and HIV-1_{U31} have very different sequences, KRIHIGPGRAFYTT and RQRTPIGLGQALYTT, respectively, around the apex of the V3 loop. The peptides used in this work were based on these sequences which differ at 8/14 positions: the differences at the loop apex, GPGR and GLGQ, respectively, may be especially important as it has been proposed (Bruce

et al., 1993) that the presence of a leucine residue instead of the proline at the apex would not favour the formation of a type II β-turn, predicted from the algorithm of Wilmot and Thornton (Wilmot & Thornton, 1988) for the classic GPGR tip motif which is present in many Ugandan variants as well as in subtype B viruses, but could allow the formation a distorted type I β-turn which would impart a different secondary structure to the V3 loop. Many Ugandan viruses have a V3 loop without a proline residue at the apex (Oram *et al.*, 1991; Albert *et al.*, 1992; Myers *et al.*, 1993) and these would be expected to induce antibodies which are not recognized by peptides based on consensus sequences: I found that 7% of the 1985/6 sera and a surprisingly high 20% of the 1990/2 sera did not react with any of the peptides used in the assays; the difference was statistically significant ($p<0.05$). These data indicate that a relatively high proportion of seropositive persons were infected by viruses with V3 loop sequences which were either poorly immunogenic or not closely related to the consensus sequences.

The effect of geographical factors on antibody specificity was examined by comparing the reactivities of sera from Entebbe/Kampala with those from the Masaka area, which is 80 miles southwest of Kampala. There were no significant differences in the reactivities of the two groups of sera (data not shown).

My data also shows that the V3-specific antibody repertoire is greater in AIDS patients than in asymptomatic persons. A relatively high proportion (25%) of the latter appeared to react with only one the peptides used in the assays and most of these sera reacted with either the MN or UG A consensus peptides. In a sub-group of 54 asymptomatic mothers 18 (33.3%) were singly-reactive and 12 (two thirds) of these reacted with the UG A consensus. However, DNA sequence data (personal communications from D Yirrell, P Kaleebu and JD Oram) show that almost two thirds of Ugandan virus are in subtype D and only about a third are subtype A viruses. Obviously, the two sets of data, serological

and nucleotide sequence, lead to very different conclusions about the distribution of viral subtypes in Uganda.

Using sequential samples from the Natural History cohort in rural SW Uganda, I found that the peptide binding activity of plasma samples from an individual showed gradual decreases with time in peptide binding activity to one or more of the V3 loop peptides. In some cases this resulted in a marked reduction in the reactivity with the MN loop peptide from the (presumably) high sero-conversion level. These results do not explain the high reactivity of Ugandan sera with the MN loop peptide but do show that the length of the post infection period can strongly influence this kind of serological data and thus reduce its diagnostic value. More direct data about MN loop reactivities were obtained by injecting rabbits with V3 loop peptides and these results are discussed later.

I attempted to improve the specificities of the peptide-based ELISA by the use of competitive inhibition assays, in which the sera were pre-incubated with peptides before reaction with peptide-coated assay plates. Although these assays would be more complex and time consuming than the simple ELISA, they should be well within the technical capacity of experienced and well managed laboratories in developing countries.

This was reasonably successful for some sera - for example, results obtained with sera U5055 (subtype D) showed that antibody binding to the MN or UG D peptides was specifically inhibited by pre-incubation of the sera with relatively low concentrations of the MN or UG D peptides, respectively: similarly, antibody binding to the U31 peptide was specifically inhibited by pre-incubation with that peptide (data not shown). However, more ambiguous results were obtained with other sera. Thus, the peptide binding activities of serum ACP 37026 (subtype A), which reacted with all the peptides tested, were all inhibited by pre-incubation with the autologous peptide and were also strongly but non-specifically inhibited by pre-incubation with the UG A

peptide. Hence, it appears from these data that although competitive peptide binding assays might enhance the reaction specificities of some sera, they would not be of practical use for many other cross-reactive sera.

Since these results were published (Smith *et al.*, 1994), Kaleebu and his colleagues attempted to improve the discrimination between sera which cross-reacted with V3 loop peptides encoded by the main HIV-1 subtypes (A, B, C and D) then thought to be present in Uganda although we now know that B and C subtype viruses are extremely rare in Uganda (personal communications from D Yirrell, P Kaleebu and JD Oram). Kaleebu (1995) found considerable cross-reactivities in binding to subtype A and C peptides and also between the subtype B and D peptides. The competitive ELISA gave some improvement in subtype discrimination but it was still doubtful whether it could be used as a routine method for sero-surveillance. Barin *et al.* found that by introducing a peptide blocking stage, in a competitive inhibition assay, they were able to give a more specific EIA which could be used at different disease stages. However, sera from the Central African Republic were still cross-reactive with V3 loop peptides, including subtype B, which led to inaccurate serological subtyping (Barin *et al.*, 1996).

One of the problems in the use of V3 loop peptides in serological assays is the lack of knowledge about the epitopes recognized by human antibodies. The V3 loop region varies in length between about 35 and about 39 amino acid residues and appears to encompass several epitopes recognized by monoclonal antibodies derived from humans and animals (Korber *et al.*, 1995). Hence, it is probable that the position and length of the peptide used in assays could be just as important as its' composition. A further problem is that short peptides adsorbed or coupled to a solid surface may adopt a very different conformation than that presented *in vivo*. This is especially true in the case of the V3 loop which is thought to adopt a hairpin structure facilitated by the apical β-turn between the disulphide bridge.

I addressed this question by comparing data obtained by reacting sera with peptides spanning the whole loop of the UG D consensus sequence with three sub-regions - the N-terminus, the apex and the C-terminus. It was intended make a similar comparison using equivalent peptides derived from the MN peptide but, due to technical problems (referred to in Section 3.2.4), it was not possible to obtain the C-terminal peptide.

In a rather limited study containing 13 sera, I found that whereas nearly all Ugandan sera reacted with the whole loop MN, UG A and UG D peptides, only one serum reacted with the whole loop U31 peptide and one serum which reacted with the apex 16-mer peptide did not bind to the whole loop peptide. There were also discrepancies between the binding of other sera to the whole loop MN, UG A and UG D peptides, some binding to the whole loop peptide and not to the 16-17-mer apical peptides and *vice versa*. Further discrepancies were found between binding to 8-mer and 16-17-mer peptides: not surprisingly, many more sera bound to the larger than to the shorter MN and UG D apical peptides (100% versus 30% and 70% versus 15%, respectively).

Almost all the sera reacted with the UG D C-terminal peptide, indicating that this region accounted for most of the reactivity observed for the whole loop peptide. Since these sera gave similar reaction patterns with the MN and UG A whole loop peptides, it is probable that most of these reactions were with an epitope in the C-terminal part of the loop.

The C-terminal regions of the whole V3 loop used were:

MN:	GRAFYTTKNIIGTIRQAHC
UG A:	GQTFYATGEIIGDIRQAHC
UG D:	GQALYTTNIIGDIRQAHC
U31:	GQALYTTKGRGTTKVIGQAHC

The MN and UG D peptides have very similar C-terminal amino acid sequences and the UG A peptide shares the last 10 amino acid residues with the UG D peptide.

MN:	YTTKNIIGTIRQAHC
UG D:	----.---D------
UG A:	-A-GE---D------

The U31 loop contains a three residue (TTK) insertion near its C-terminus, which would be expected to give a different conformation to this part of the loop.

Hence, it seems possible that part of the cross-reactions observed between some Ugandan sera and the MN peptide could be due to shared C-terminal epitopes. However, much of the work presented in this Thesis was done using shorter (16-19mer) peptides which included only 8-11 amino acid residues C-terminal to the loop apex so it seems probable that there must be at least one shared or cross-reactive epitope fairly close to the apex. This is consistent with data obtained in other studies including, for example, the demonstration by Meleon *et al.* that many HIV-reactive human and chimpanzee sera recognized a 9-mer peptide spanning the apex and that the sequences immediately C-terminal to the apex were important for the specificity of antibody binding (Meleon *et al.*, 1989). Several monoclonal antibodies have also been found to bind to this region (Korber *et al.*, 1995).

These data draw attention to some of the problems associated with the use of simple peptides in serological assays and emphasize the crucial importance of the selection of the peptides and the standardization of the assays in different laboratories, for which there seems to be little enthusiasm, at present.

One of the major problems in the interpretation of ELISA data for sera from HIV-infected persons is that, except in exceedingly rare cases, the exact sequence of the immunogen is unknown. Under these conditions it is not possible to determine whether cross-reactive antibodies against an epitope reflects a range of antibodies induced by sequential variants of that present in

the infecting virus or whether it is due to the induction of antibodies against shared epitopes or with a relatively low specificity. The data I obtained by vaccinating rabbits with whole V3 loop peptides is relevant to these questions. The data showed that whereas the MN peptide was a very good immunogen, inducing high binding titres towards the autologous peptide, it also induced relatively high titres against the UG A and UG D peptides; a very low titre of reactivity towards the U31 peptide was obtained in one rabbit vaccinated with the MN peptide. Competitive inhibition assays indicated that the MN, UG A and UG D reactive antibodies were specific for the MN and UG A peptides.

The U31 peptide was a relatively good immunogen, producing high titre U31-specific antibodies in 2 out of 4 rabbits. However, both the UG A and UG D peptides were poorly or non-immunogenic in 7 out of 8 rabbits. I conclude from these peptide-binding data that the two consensus peptides were not very immunogenic but this is contradicted by the virus neutralization data for these sera (see below).

It appears from these results that an immunogenic peptide such as the MN whole loop peptide can induce high titres of antibodies which cross-react with similar loop structures but not with those with less conserved structures like, for example, the U31 loop. By extrapolation to infected humans, it would seem likely that the virus, which would probably have a relatively invariant V3 loop structure at the sero-conversion stage (Zhang *et al.*, 1993) would also induce antibodies with a wide spectrum of specificities, including cross-clade reactivities. This would make it very difficult to develop a subtype or sequence specific V3 loop peptide-based assay which would be of general use. However, it might be possible to develop a more specific assay for use in restricted situations, for example, with incident cases in a small community or cohort where the viruses may have a relatively narrow range of V3 loop sequences. Further, peptides based on common sequence motifs may show less cross-reactivity than those derived from consensus sequences. However, this is far from certain, in view of the wide cross-reactivities I found in sera induced by single well defined peptides in rabbits.

4.2 Virus Isolation and Characterization

The second main objective of my work was to investigate the neutralization of Ugandan viruses by autologous and heterologous sera, including those from persons infected with viruses in other subtypes. At the time (1992) when this work was started most laboratories were studying the neutralization of cell line adapted viruses which seemed to have little relevance to virus control or protection *in vivo*. Although many cell line adapted Ugandan viruses were available in the CAMR Repository, which also contained many HIV-infected primary PBMC samples, these were all taken in 1985/6 and it was felt that I should work with more recent primary isolates. With the help of Dr B Tegume, Dr B Biryahawaho and Dr JD Oram, blood samples were obtained from persons attending the UVRI AIDS clinic in 1992. These samples were fractionated at UVRI and the plasma and leucocyte fractions dispatched in liquid nitrogen to CAMR, where I attempted to isolate viruses by co-cultures in PBMC.

The rate of virus isolation was relatively low - 14 out of 30 samples, i.e. 44%, but an even lower isolation rate (21%) was also obtained for Ugandan viruses by the WHO Network for HIV Isolation and Characterization (1994). This group blamed operational and logistical difficulties in collection and shipment of blood samples to collaborating laboratories but this was not a problem in the samples I received. They also quoted a personal report from Dr Leitner and Dr Albert that they were only able to isolate 2/11 viruses from Ugandans living in Sweden. Much better isolation rates were obtained by Dr Desselberger and his colleagues at the Cambridge Public Health Laboratory for samples obtained from the MRC in Uganda: many of these isolates were subsequently used in this investigation.

The majority of the viruses isolated from the UVRI clinic samples had the SI phenotype although, as far as is known, the majority of the persons bled were either asymptomatic or had only minor disease symptoms. Unfortunately, many of the persons attending the clinic do not re-appear for further sampling or counselling and their clinical and serological records are somewhat

fragmentary. As evidenced by the differences in phenotypes of three isolates in PBMC and in cell lines, several of the blood samples appeared to contain a mixture of NSI and SI viruses, possibly indicating a phenotypic transition of the viruses in the infected persons concerned. A similar high proportion of SI viruses in primary isolates was described by Simmons *et al.* (1996). I also found that one of the viruses which had the SI phenotype in PBMC did not produce syncytia in the T cell lines and that some NSI variants can grow quite well in T cell lines and may even be selected in the presence of SI variants.

I found that many of the isolates gave rather low titres, mostly between 10^1 and 10^3, in both PBMC and MT-2 cells. These titres are very similar to those since reported by Simmons *et al.* (1996) for primary SI and NSI isolates and I found that for some isolates the amounts of virus were sufficient for virus neutralization assays.

Whilst there is at present little data to indicate that neutralizing antibodies have a protective role against HIV infection, it would be very unwise to ignore their possible importance. It is unlikely to be a co-incidence that the huge decrease in plasma virus occurs post seroconversion at a time of maximum anti-HIV antibody production and when the HIV-specific CMI responses are also well developed. Therefore, it is not possible to define the separate roles of CMI and neutralizing antibodies in the control of virus and the maintenance of the virus/host equilibrium during the asymptomatic phase of infection. This virus-induced "immunity" can persist for many years or perhaps, in a few individuals, for life. Hence, it is important to characterize both the cellular and humoral immune responses to infection by HIV and to harness our understanding of the combined protective responses towards the production of effective vaccines against HIV. In view of the urgent need for a vaccine for HIV in Uganda and the surrounding countries, it is especially important to investigate immune responses in Africans infected with locally dominant subtypes of virus.

4.3 Virus Neutralization

The main objectives of the virus neutralization studies which formed the larger part of my work were:

Firstly, to produce quantitative data, including neutralizing antibody titres and specificities, of human sera against primary Ugandan isolates.

Secondly, to determine whether neutralizing antibodies were effective against viruses from different subtypes.

Thirdly, to investigate possible correlations between peptide binding reactivities and neutralizing activities in human sera.

Fourthly, to measure the neutralizing titres and specificities of antibodies produced in rabbits vaccinated with whole V3 loop peptides.

Finally, to examine the possible protective role of neutralizing antibodies in a Ugandan cohort.

My initial neutralization experiments were done using viruses isolated from patients attending the UVRI clinic in Entebbe. However, as described above, it was difficult to obtain follow up samples and data from most of these patients and therefore the main part of this work was done with primary virus isolates from the Natural History cohort in rural SW Uganda, as these became available from the MRC AIDS Reagent Project in 1993/4. The availability of the blood samples from this cohort enabled me to isolate viruses and antisera as paired samples. The samples with sufficient quantity of antiserum to allow replication of experiments were used for neutralization studies. This cohort has been intensely studied over the past eight years by the MRCPA in Uganda Programme and a large body of clinical, serological and T cell sub-set data has been produced. This cohort seemed an obvious choice for studies on neutralization specificities both within and between virus subtypes and the role of neutralizing antibodies on the rate of disease progression.

These viruses were available as low passage (some were also low titre) isolates with matching plasma. In addition, the viruses had been phenotyped using PBMC and T cell line cultures by Gill Carnegie and most had been genotyped for both *gag* and the gp41 region *env* gene sequences by Catriona Baker, both

working in Dr Desselberger's Laboratory in Cambridge. I am very grateful to Dr Desselberger and his colleagues for making their data available prior to publication. A further advantage was that it was possible to obtain sequential serum samples and to obtain fresh blood samples for sequential virus isolation work. I am very grateful to Mr Amato Ojwiya for supplying serum samples from the MRCPA Repository in Entebbe, to Dr Dilys Morgan for arranging the collection and transport of the fresh blood samples from the Natural History cohort in rural SW Uganda and to Dr Benon Biryahawaho for allowing me to use the UVRI Virology laboratories to fractionate these blood samples in Entebbe.

My first neutralization experiments were done using MT-2 cell adapted viruses, since the assays were technically easier than those using primary isolates. The main problem with assays using primary isolates was the maintenance of viable cultures of PBMC in microtitre plates over a 10 day period. However, with the kind help of Dr Pontiano Kaleebu, this problem was solved within a fairly short time and I was then able to compare neutralization data obtained with MT-2 cell adapted and primary isolates. This was done for three Ugandan viruses, obtained from the UVRI clinic, and also for two different preparations of HIV-1_{MN}; back-adapted to grow in PBMC and adapted to grow in MT-2 cell culture. Whereas there was a reasonable correlation between neutralization titres obtained with the two HIV-1_{MN} viruses, the correlation was much worse for the Ugandan isolates: the primary isolate of one of these was neutralized by all seven human sera tested but the corresponding MT-2 cell adapted virus was only neutralized by two of these sera with titres >20. These results raise a question about the validity of neutralization data obtained with cell line adapted viruses - which are still being used to monitor immune responses in some vaccine trials. One may also question the validity of neutralization assays done with primary isolates (selected by culture *in vitro*); however these experiments are at least one stage closer to the *in vivo* situation. *In vivo* assay systems, using unselected plasma virus or virus infected cells possibly within the patient, have

yet to be developed and would in all probability be either completely impracticable or unethical.

The main conclusions from my studies on the neutralization of primary isolates is the majority of sera from infected persons neutralized a fairly large number of viruses, and many had relatively high neutralization titres - over 640 in many of the Natural History cohort plasma. These results were unexpected as most of the previous studies with primary isolates reported relatively low titre neutralizing antibodies which in some cases were effective against a restricted number of viruses (Trkola *et al.*, 1995; Kostrikis *et al.*, 1996; Mascola *et al.*, 1996a; Moore *et al.*, 1996). However comparison of my results with some work by Kaleebu with Ugandan isolates revealed similar levels of p24 and neutralizing antibody titres (Kaleebu, 1995). The neutralizing antibody titres found by Moore *et al.* (1996) for Ugandan subtype A and D isolates were shown in many cases as >128, where 1:128 was his highest serum dilution. The neutralizing antibody titres found by Kostrikis *et al.* (1996) for subtype A isolates were very similar to my titres against primary isolates. Only two (C24 and UG92001) out of the 18 viruses that I tested were not neutralized by their autologous serum: unfortunately, due to the very small volume of serum available, it was not possible to test this serum C24 against a range of viruses so this atypical result could not be proved rigorously. Serum UG92001 neutralized 4 other Ugandan subtype D viruses with titres ranging from 40 to >320 and HIV-1_{MN} subtype B with a titre of >320.

Many of the sera neutralized all the primary isolates tested, i.e., 8/8, 9/9, 10/10, etc, and several others neutralized all but one or two of the viruses tested. However, a few sera neutralized only one or two viruses: for example, serum UG92001, from Entebbe/Kampala, neutralized only 4 out of 7 viruses tested and serum 3004, from the Natural History cohort neutralized only 2 out of 10 viruses isolated from the cohort. A similar correlation between primary isolates' susceptibility to neutralization by autologous and heterologous antisera has been reported by Bongertz *et al.* (1997).

The viruses used in this work, especially those obtained from the Natural History cohort in rural SW Uganda, were not positively selected and are reasonably representative of those circulating within the local population. Therefore, these neutralization data show that most infected persons mount an antibody response which is able to neutralize many of the viruses which are circulating in their community. This has good implications for the prospective protective effects of neutralizing antibody responses which could be induced in the majority of vaccinees especially if the vaccine included immunogens from several different viruses.

Although most of the primary isolates were very sensitive to neutralization by one or more of the sera tested, being neutralized by dilutions of sera typically between 1:10 and 1:320 or 1:640, one or two were more resistant. For example, the neutralization titres against virus UG93070, from the Entebbe/Kampala area, ranged between <10 to 40, in marked contrast to those obtained with other isolates. The reasons for this variation in sensitivity are not known. Von-Gegerfelt *et al.* (1991) observed that some viruses isolated a year or two after seroconversion were no longer neutralized by sera taken previously and argued that this showed that new, neutralization-resistant variants had arisen in the interval between sampling. It was therefore of interest to compare the susceptibilities of sequential isolates from the Natural History cohort in rural SW Uganda, taken about 2 years apart, to neutralization by sera taken over the same period. The two isolates of virus 3049 were almost equally sensitive to neutralization by the four autologous plasma: these sequential plasma also had essentially the same titres against several other virus isolates, indicating that there was little qualitative or quantitative change in virus sensitivity or neutralizing activity over the two year period. The two virus 3029 isolates were equally sensitive to neutralization by autologous sera, but the later virus was much more resistant towards heterologous sera. Hence, although I found no evidence for the appearance of resistance towards autologous sera, one of the two viruses appeared to have become much more resistant to other sera. Clearly, more data involving more viruses for a much longer period is

required to show if this is a general rather than isolated phenomenon and to assess its biological/immunological importance, if any.

These neutralization studies were started in 1993 when it was not known whether viruses neutralization would be subtype specific. Since then there have been a few reports that neutralization of isolates by human or animal sera is not subtype specific (Trkola *et al.*, 1995; Mascola *et al.*, 1996a; Moore *et al.*, 1996). An extensive study involving primary isolates from all of the 9 subtypes of the major group of HIV-1 identified five neutralization "serotypes" which, with the exception of the subtype E isolates from Thailand, did not correlate with the genotypes of the isolates (Kostrikis *et al.*, 1996).

My data shows that Ugandan isolates in subtypes A and D are cross neutralized by sera induced by virus of either subtype, indicating a set of shared immunogenic epitopes which is potentially valuable in vaccine development. However, sera GB8 from a subtype B virus, obtained from an AIDS patient in the UK, neutralized only 3 out of 8 Ugandan isolates (2 subtype D's and 1 of unknown subtype) and had very low titres (≤15) against two of those. Moreover, a monoclonal antibody induced by a V3 loop peptide encoded by HIV-1_{MN} (Gorny *et al.*, 1991) neutralized only one out of 8 Ugandan isolates. Hence, in view of the low level cross neutralization of some subtype B sera for subtype A or D viruses, it could be very important that a vaccine for use in Uganda should be based on subtype A and D viruses and not, as is the case for a vaccine currently undergoing trial in Uganda, on an American subtype B virus. However, the converse situation, that a vaccine based on subtype A and D viruses may not be effective against viruses in other subtypes, would probably not be of concern in Uganda. Although there have been reports (Bruce *et al.*, 1993; Kaleebu *et al.*, 1995 and C Baker, MA Thesis, University of Cambridge) of the presence of subtype B, C and G viruses in Uganda, only subtype A and D proviruses were detected by *gag* sequence analyses of over 120 proviruses from the MRC cohorts (Yirrell *et al.* - Submitted for publication (1997) and Kaleebu *et al.*, 2000).

A possible complication in the development of vaccines for HIV is that the vaccine may induce virus-enhancing antibodies which could accelerate the rate of virus replication. I found some evidence for enhanced virus production in six out of 25 Ugandan isolates. For example, in one experiment with UG92001, a virus isolated from a UVRI clinic blood sample, virus yield was increased by 28% by antiserum C971 at 1/80 dilution. The yield of another virus, 3050 from the Natural History cohort in rural SW Uganda, was enhanced by between 20% and 70% by relatively high dilutions (1/80 to 1/320) of four antisera. Since all sera were routinely heat-treated before use, it is unlikely that these effects would have been due to the kind of complement-dependant enhancement effect reported by (Robinson *et al.*, 1988a). As I did not investigate the effect of added complement on virus neutralization, my data does not show whether enhancing antibodies were widespread in the Ugandan sera.

Comparison of the serological data with the corresponding virus neutralization patterns indicated that there was no clear cut relationship between the two types of reactivity. For example, several of the plasmas obtained from the UVRI clinic did not react with the U31 V3 loop peptide but neutralized the HIV-1_{U31} primary isolate, with neutralization titres which were not significantly higher than that obtained with plasma which reacted strongly with the U31 peptide. Further, plasma UG92035, which did not react with any of the V3 loop peptides tested, neutralized 6 out of the 8 viruses tested. Data obtained with plasma/virus samples obtained from the natural history cohort in rural SW Uganda also showed there was no correlation between serotype and neutralization phenotype. Hence, my data indicates that, for many of the plasma/virus interactions, at least part (and possibly a major part) of the neutralizing activity was not directed against the linear V3 loop epitopes.

I also attempted to correlate the virus neutralizing activities of antisera produced by vaccinating rabbits with whole V3 loop antibodies with data obtained in peptide-based ELISA. The MN peptide induced the highest titred antibodies in both the serological and virus neutralization assays. However, the U31 peptide induced a relatively good peptide-specific serological response but only a very weak neutralizing activity and did not neutralize the autologous virus. The sera induced by the UG A consensus peptide reacted strongly with the MN peptide and neutralized the back-adapted HIV-1_{MN} and 2/8 Ugandan isolates. However, the UG D consensus peptide induced sera which reacted poorly with peptide antigens but neutralized several Ugandan isolates. Hence, these experiments gave conflicting results, ranging from a good correlation for the MN peptide and a very poor one for the U31 peptide. Taking these uncertainties and those obtained in studies with human sera into account, it would probably be unwise to base predictions of virus neutralizing specificities or titres on data obtained in peptide binding assays with V3 linear epitopes.

4.4 Correlation of Clinical and Virus Neutralization Data

One of the main objectives of this study was to determine whether the presence of neutralizing antibodies could influence the rate of disease progression in the MRC Natural History cohort in Uganda.

As discussed earlier, persons in the cohort are tested at 3 monthly intervals making it ideal for this type of study, especially since much work on virus isolation and characterization had been done in UVRI at Entebbe and in the Public Health Laboratory at Cambridge. However, there were a number of problems in attempting to correlate the clinical, virological and virus neutralization data. For example, it was only possible to study a fairly limited number of viruses. I worked with 14 out of the 32 viruses isolated at Cambridge, but 4 of these grew very poorly in PBMC: hence, it was not possible to get neutralization data for these viruses. In addition, relatively few persons infected with these viruses developed AIDS, by which disease stage

the immune system would be impaired and unable to maintain humoral and cellular immunity. Although the exact date of seroconversion is not known, two of the persons included in my study were infected in late 1990/early 1991 and the other 12 between early 1992 and early/mid 1993. Clinical data was obtained for the 3 year period up to early 1997, by which time most of the people had been infected for between 4 and 6 years. At that time Morgan *et al.* (1997) reported that in this cohort the mean time from infection to AIDS was 4.4 years and to death was 6 years. From their data it would be expected that the majority of the subjects included in my study would have developed AIDS but, in fact, only two of the 14 persons had progressed beyond WHO stage 3. Both of these persons died - one about 3-4 years after infection; unfortunately, it was not possible to obtain clinical data for the other person who died. Although six of the other 11 persons had progressed to WHO stage 3 within the study period, it would not be expected that they would have shown a marked loss of virus neutralizing activities at this stage.

Excluding the one person who died for whom clinical data was available, three others showed a marked decrease in their CD4 counts, two of whom were at WHO disease stage 3 and one at stage 2. As one of the viruses from the stage 3 patients was not tested with the autologous plasma and the other was not tested with heterologous plasma, full neutralization data was obtained only for the latter, whose plasma had a relatively low titre against the autologous virus and high titres against heterologous viruses.

A further problem was that, with two exceptions, the viruses tested were isolated relatively soon after infection, i.e. within two years. Since there is good evidence (Von-Gegerfelt *et al.*, 1991) that sequential virus isolates may not be neutralized by preceding sera, it would have been more relevant to have studied viruses from later samples from the Natural History cohort in rural SW Uganda. I was able to do this for two viruses, which I isolated two years after their first isolation at Cambridge. Both of these were from persons who showed a marked decline in their CD4 cell counts; however the two isolates gave rather conflicting data. All but one of the sequential plasma

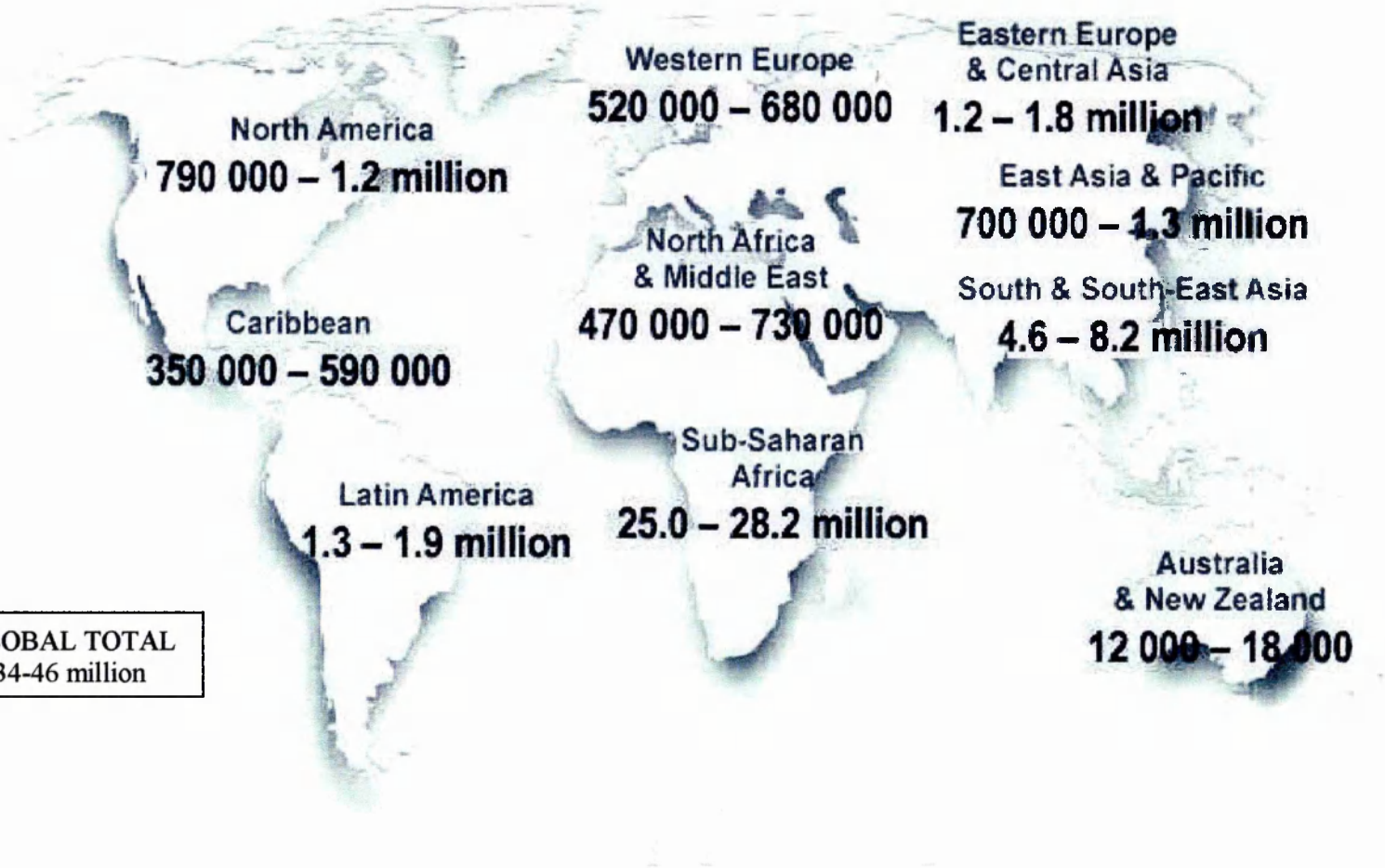
samples (taken over the same two year period) gave similar titres against the early and late isolates of the two autologous viruses. However, although one of the later isolates was much more resistant to neutralization by heterologous plasma, the other was still fairly strongly neutralized by those plasma which neutralized the early isolate. Hence, there were no clear cut differences between the early and late isolates that could be correlated to change in disease status, as shown by the drop in CD4 cell counts. In summary, I obtained insufficient data in this rather limited study to test thoroughly the possible correlation between the clinical and neutralization data.

It might be expected that the development of a strong virus neutralizing antibody response to HIV infection would help protect against disease and death. I attempted to see if there was any evidence that neutralizing antibodies have a protective effect from the comparison of neutralization data and the clinical data available from samples from the Natural History cohort. Analysis of the change in the CD4+ cell count with the autologous virus neutralizing titres for 8 samples for which both data were available (as shown in Table 14 and Figure 20) was insufficient to determine a relationship between the presence of autologous virus neutralizing antibodies and disease progression.

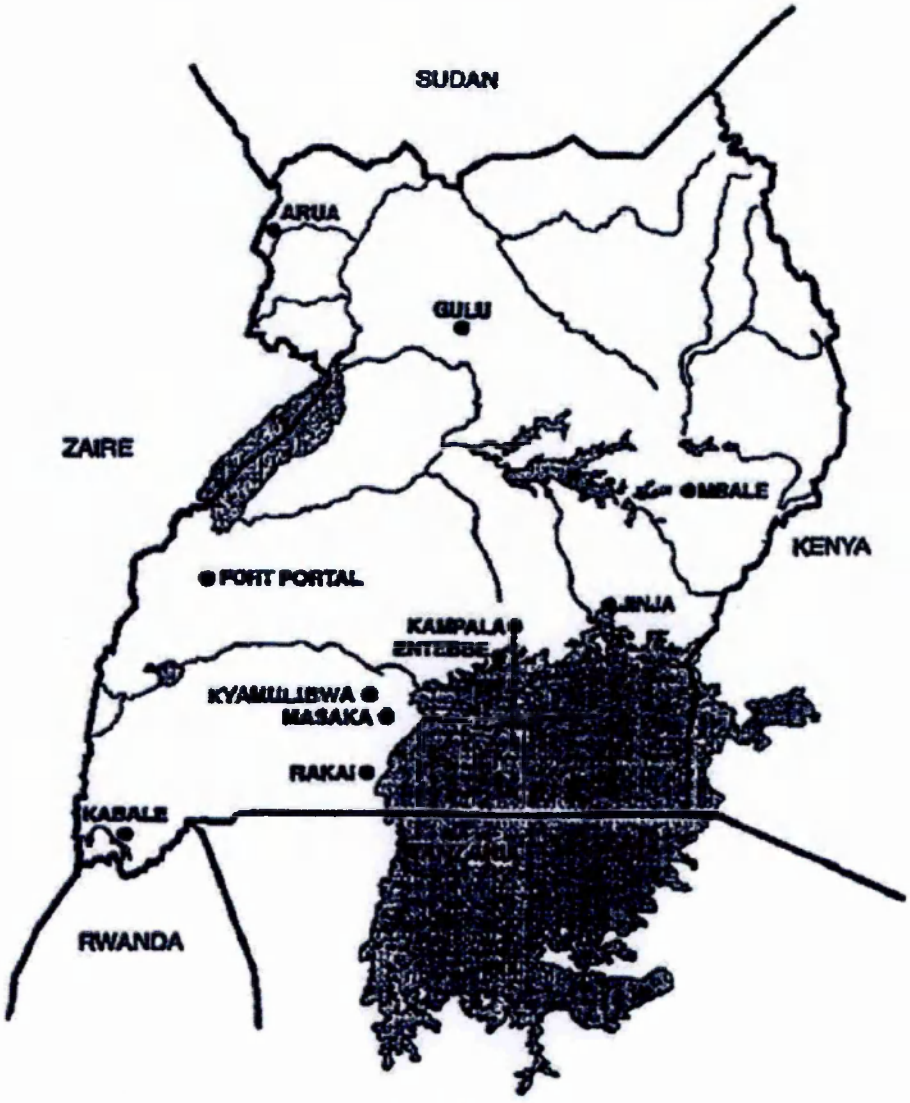
Clearly, additional neutralization data obtained from sequential plasma and, if possible, later virus isolates, would be required to extend these studies in future for a more meaningful test of the possible correlations between disease progression and the presence of neutralizing antibodies. These data would be especially useful in cases where individuals have progressed to AIDS.

I hope that these preliminary studies may be extended, especially as the cohort is still being followed and the MRCPA and UVRI have all the necessary facilities and expertise.

Appendix I: Estimated number of persons living with HIV/AIDS at the end of 2003 (Adapted from UNAIDS and WHO AIDS Epidemic Update: December 2003).



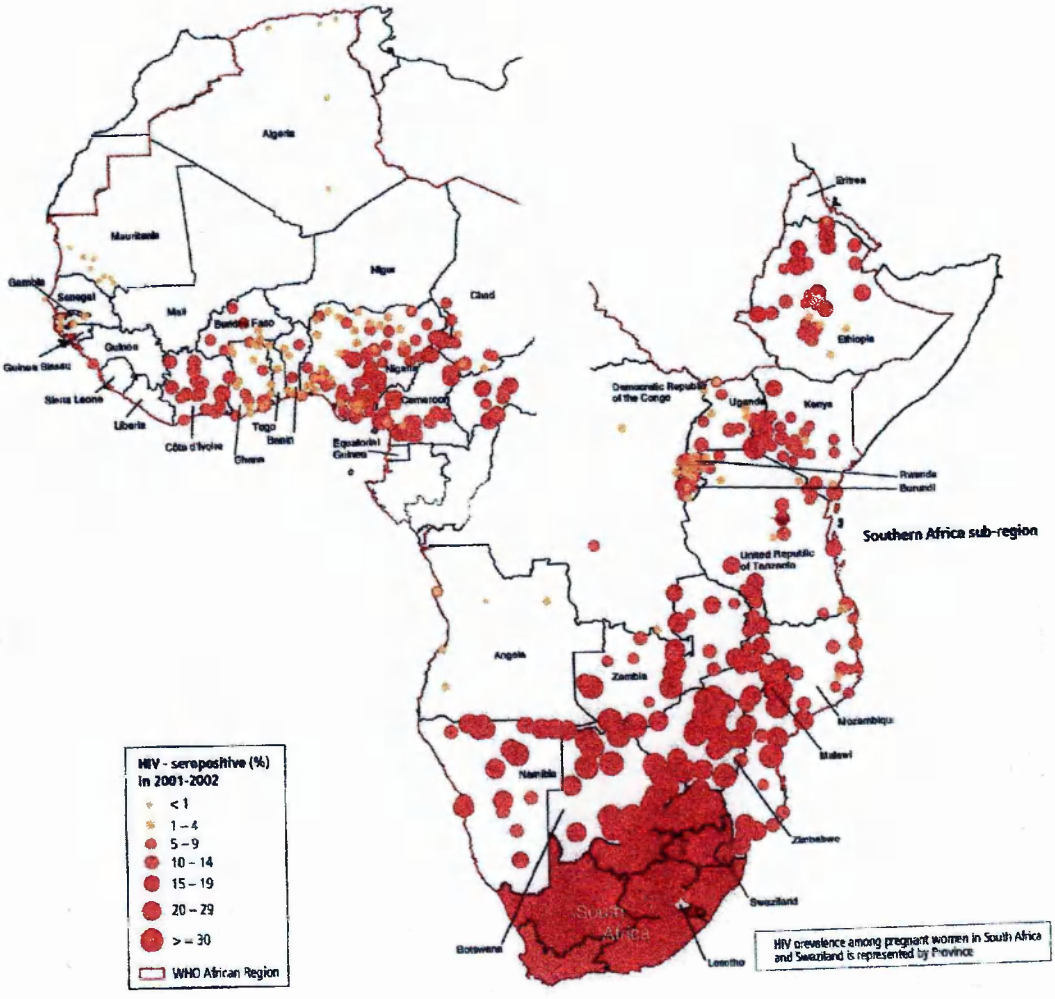
Appendix II



Map of Uganda

Appendix III

HIV prevalence among women attending antenatal clinics in Africa. (Adapted from the UNAIDS and World Health Organization HIV/AIDS Epidemiological Surveillance Update for the WHO African Region 2002.)



APPENDIX IV

Materials List

Antisera, plasmas, viruses and peptides were as described in the Materials and Methods text.
All chemicals mentioned in the text, but not listed below were obtained from The Sigma Chemical Company.
Antibody binding assay 96 well plates - Nunc, Maxisorb (high affinity binding).
Dilution 96 well plates - Corning, Delta (low affinity binding).
Foetal Calf Serum - ICN Flow.
Anti-human IgG-horse radish peroxidase conjugate - Sigma.
Anti-rabbit IgG-horse radish peroxidase conjugate - Jackson Immuno Research Laboratories Inc..
3,3',5,5'-tetramethylbenzidine dihydrochloride tablets - Sigma.
Activated CH-Sepharose - Pharmacia.
Keyhole Limpet Haemocyanin - Sigma.
PD10 column (Sephadex G-25 medium column) - Pharmacia.
Sephadex G-50 fine column (1.6 x30cm) - Pharmacia.
Freund's Incomplete Adjuvant - ICN Flow.
Dutch modified RPMI 1640 cell medium - ICN Flow.
Ficoll-Paque - Pharmacia.
Centrifuge tubes (50ml and 15ml) - Corning.
Wide bore pastettes - Elky Eireann.
Kanamycin - Sigma.
Phytohaemagglutinin (PHA) - Sigma.
Interleukin-2 (IL-2) - MRC-ARP.
EDTA Vacutaner tubes - Becton Dickinson Vacutaner Systems.
Cell culture flasks ($25cm^3$) - Corning.
Empigen detergent - Calbiochem Nova Biochem UK LTD.
P24 Antigen assay kit - Coulter.
Cryotubes - Nunc.
96 well round bottomed plates, tissue culture treated - Corning.

APPENDIX V

Definition of the WHO Clinical staging:

Clinical Stage 1 -

1. Asymptomatic.
2. Generalized lymphadenopathy.

Performance Scale 1: Asymptomatic, normal activity.

Clinical Stage 2 -

3. Weight loss < 10% body weight and > 5%.
4. Minor mucocutaneous manifestations.
5. Herpes zoster within the last 5 years.
6. Recurrent upper respiratory tract infections.

And/or Performance Scale 2: Symptomatic, normal activity.

Clinical Stage 3 -

7. Weight loss > 10% body weight.
8. Unexplained chronic diarrhoea > 1 month.
9. Unexplained prolonged fever > 1 month.
10. Oral candidiasis.
11. Oral hairy leukoplakia.
12. Pulmonary TB within 1 year.
13. Severe bacterial infections.

And/or Performance Scale 3: Bed-ridden < 50% of day during last month.

Clinical Stage 4 -

14. HIV wasting syndrome.
15. Pneumocystis pneumonia.
16. Toxoplasmosis of brain.
17. Cryptosporidiosis with diarrhoea > 1 month.
18. Cryptococcus extra-pulmonary.
19. CMV of an organ other than the liver, spleen or lymph nodes.
20. HSV infection, mucocutaneous > 1 month, or visceral.
21. Progressive multifocal leukoencephalopathy.
22. Disseminated endemic mycosis.
23. Candidiasis of oesophagus, trachea, bronchi or lungs.
24. Atypical, disseminated mycobacteriosis.
25. Non-typhoid, salmonella septicaemia.
26. Extra-pulmonary TB.
27. Lymphoma.
28. Kaposis sarcoma.
29. HIV encephalopathy.

And/or Performance Scale 4: Bed-ridden > 50% of day.

APPENDIX VI

BINDING OF ANTIBODIES TO V3 LOOP SPECIFIC PEPTIDES.

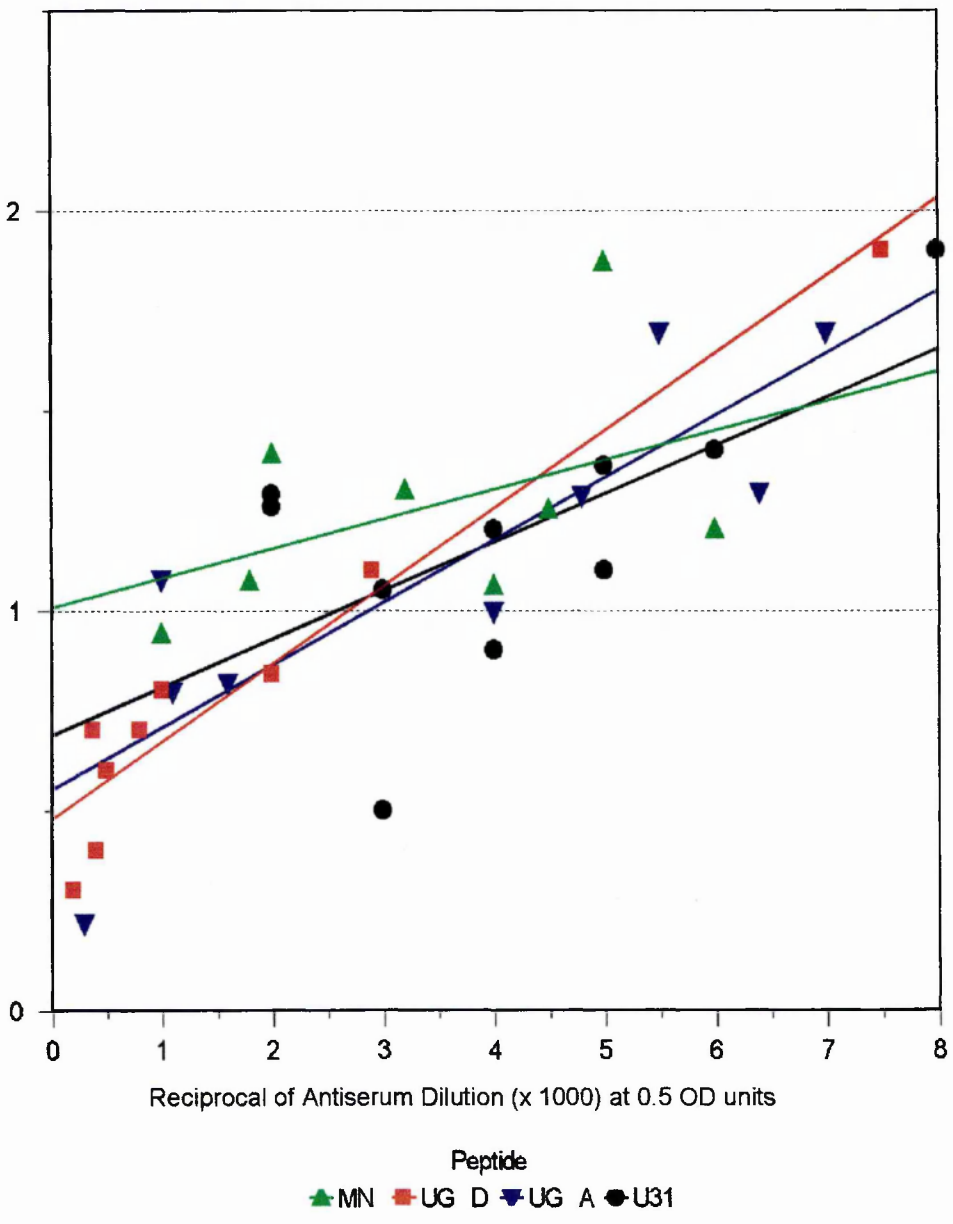
SERA (SUBTYPE)	PEPTIDES USED IN ASSAYS			
	MN (USA)	UG A (UGANDA)	UG D (UGANDA)	U31 (UGANDA)
U455 (A)	2000 1.39 / 1.38	375 0.7 / 0.78	- -	6000 1.4
U4133 (D)	5000 1.87 / 1.68	- -	4000 1.0 / 0.87	4000 0.9 / 0.92
U1685 (D)	6000 1.2 / 1.12	800 0.7 / 0.61	6400 1.3 / 1.14	8000 1.9 / 2.0
U653 (D)	4000 1.06 / 1.15	500 0.6 / 0.63	4800 1.4 / 1.29	5000 1.4 / 1.36
U462 (D)	60000 1:400 1.65 / 1.52	2900 1.1 / 1.1	1600 0.82 / 0.84	3000 1.05 / 1.12
U2999 (D)	20000 1:400 1.38 / 1.36	- -	5500 1.7 / 1.78	3000 0.5 / 0.43
U4132 (D)	20000 1:400 1.6 / 1.74	- -	- -	5000 1.1 / 0.97
U1665 (D)	5000 / 4700 1.28 / 1.39	1000 / 980 0.8 / 0.74	1100 / 1100 0.8 / 0.86	4000 / 4100 1.2 / 1.16
U5055 (D)	20000 1:400 1.93 / 1.86	2000 0.8 / 0.84	10000 1:400 1.8 / 1.77	2000 1.26 / 1.13
ACP37024 (D)	1000 / 1100 0.94 / 0.82	200 / 220 0.3 / 0.36	1000 /1000 1.08 / 1.24	- -
ACP37025 (A)	4500 / 4700 1.25 / 1.17	400 / 420 0.4 / 0.46	- -	- -
ACP37026 (A)	45000 2.08 / 2.15	7500 1.9 / 1.82	7000 1.7 / 1.56	2000 1.29 / 1.24
ACP37027	3200 / 3000 1.3 / 1.24	- -	300 / 320 0.22 / 0.26	- -
ACP38051	1800 / 1700 1.07 / 1.15	- -	- -	- -

Titres (some shown in duplicate) are reciprocals of serum dilutions giving an EIA reading of 0.5 OD units and duplicate absorbancy readings at 1:200 serum dilution are shown below these. Where indicated some absorbancy readings are at 1:400 serum dilution.

- = titre < 100 or that the absorbancy reading at 1:200 serum dilution was not significantly above the negative control.

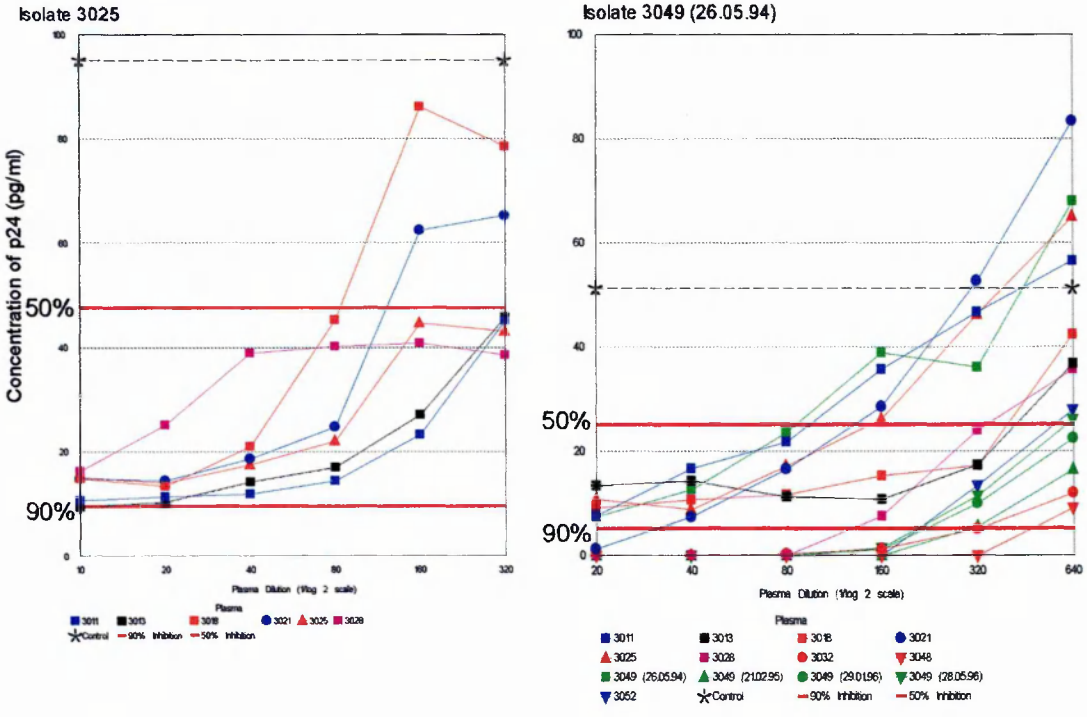
Appendix VI continued:

Figure showing the correlation between Antiserum Dilution at 0.5 OD units and the Absorbance at a single 1:200 Antiserum dilution for a range of antisera with the 4 peptides MN, UG A, UG D and U31.
Each point represents the intersection of the 2 readings for one antiserum.
The lines represent the correlation for the 4 peptides.



APPENDIX VII

This combination of data illustrates why I decided to use 75% inhibition of p24 production as my neutralization cut off point. The 2 graphs show that the 90% and 50% cut off points (indicated here by red horizontal lines) do not cross as many data lines as the 75% cut off point would. (To view these graphs in detail see Figures 16ii c/ and 16v a/.) This is backed up by the data in the table showing the antisera titres at the 3 inhibition levels for the 2 isolates. Here you can see that 90% inhibition misses the antisera that do not neutralize the isolates to this degree (shown by "-") and that 50% inhibition misses the antisera which are still inhibiting the viral p24 production to this degree at the maximum dilution (shown by ">n"). 75% inhibition of p24 production gave a varying range of titres across a broad range of antisera for many of the viral isolates.



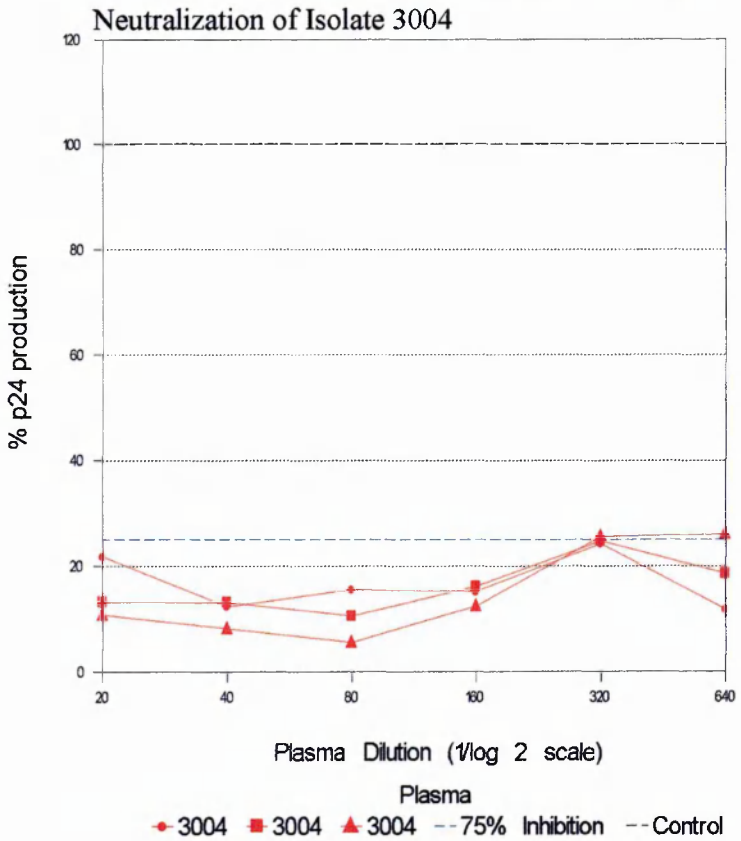
NEUTRALIZATION OF UGANDAN HIV-1 ISOLATES IN PBMC CULTURES.

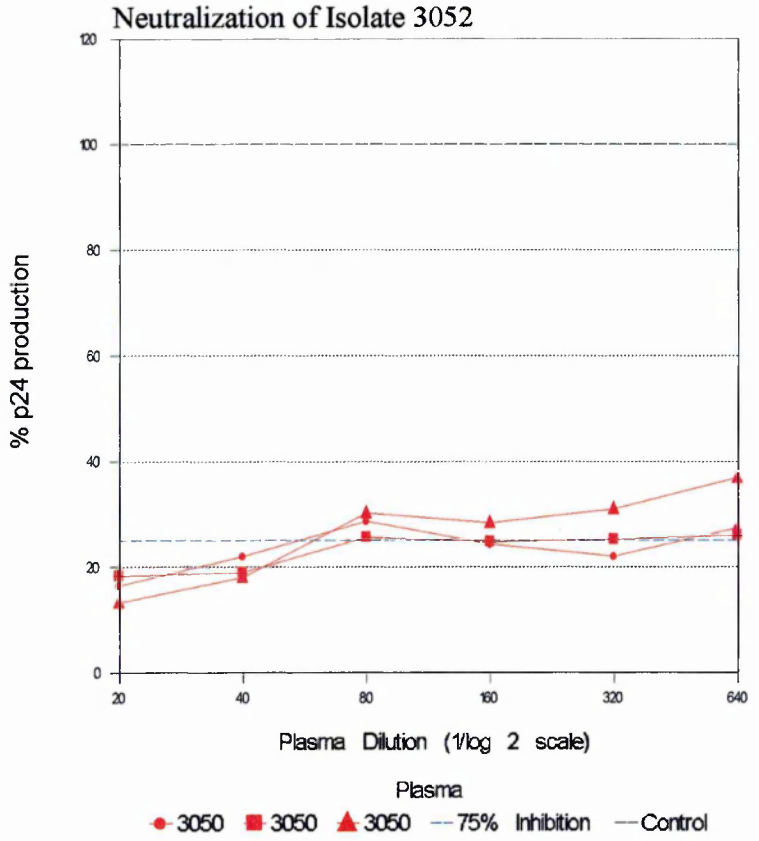
Plasma (Subtype)	Virus Isolate (Subtype) % Neutralization					
	3025 (D) 50%	3025 (D) 75%	3025 (D) 90%	3049 (D) 50%	3049 (D) 75%	3049 (D) 90%
3011 (A)	>320	160	-	80	30	-
3013 (C)	>320	120	-	340	200	-
3018 (D)	80	40	-	420	80	-
3021 (A)	120	60	-	100	60	20
3025 (D)	>320	80	-	160	60	-
3028 (A/D)	>320	15	-	320	200	-
3029 (D)	ND	ND	ND	>640	ND	100
3032 (D)	ND	ND	ND	>640	640	320
3048 (D)	ND	ND	ND	>640	>640	380
3049 (D)	ND	ND	ND	80	40	-
3050 (D)	ND	ND	ND	640	-	-
3052 (A)	ND	ND	ND	640	320	200

Figures shown as reciprocal of plasma dilution giving 50%, 75% or 90% inhibition as determined by p24 detection.
ND = Assay not done. - = Inhibition < 50%, 75% or 90%. * = *gag* subtype A and *env* subtype D.

Appendix VIII

Figures showing replication of neutralization assays for isolates 3004 and 3052 with plasma 3004 and 3050 respectively; to allow assessment of 75% neutralization titre from shallow curves.





Data expressed as the percentage of p24 produced in control wells to allow for inter-test variability.

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